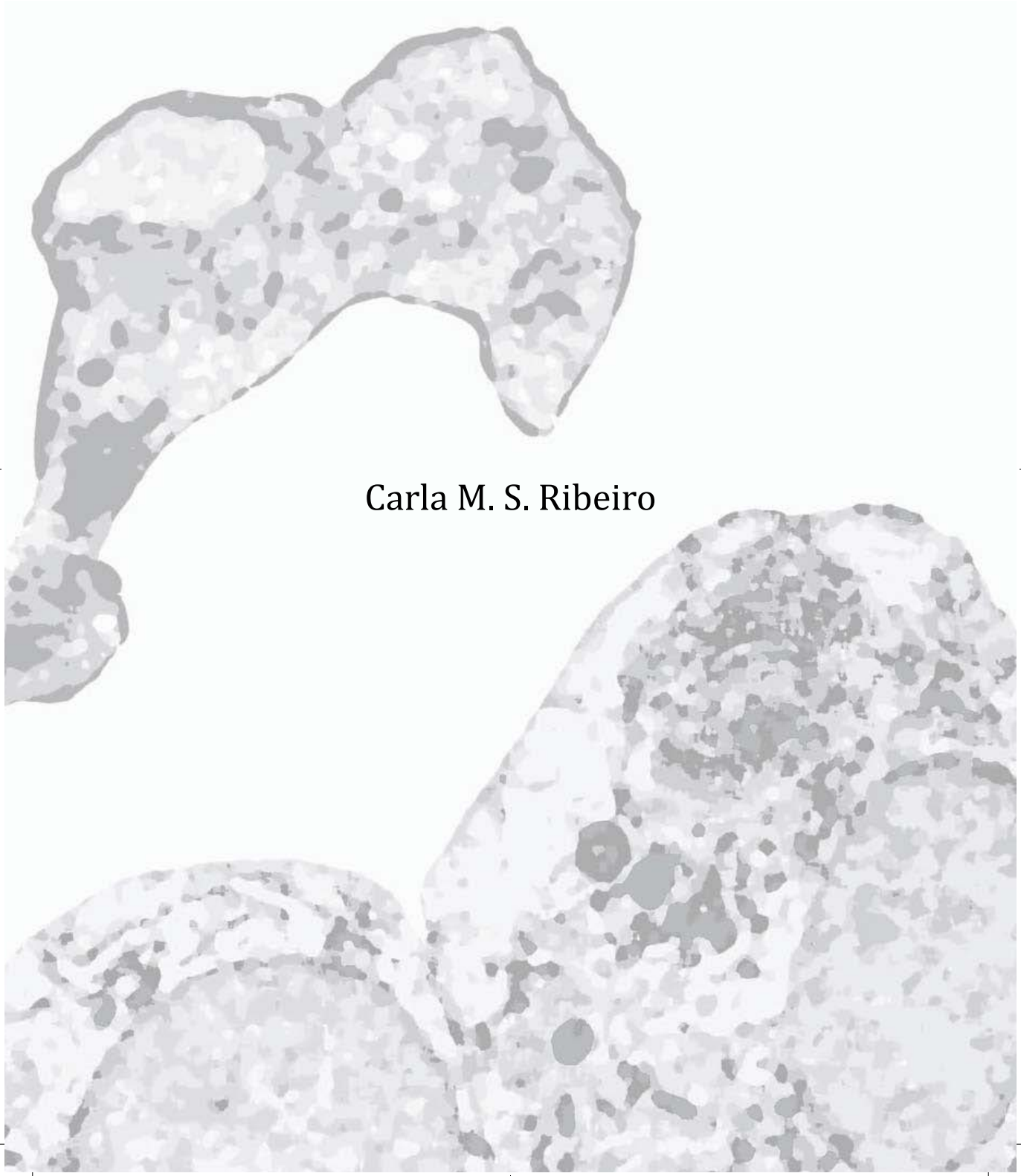
INNATE IMMUNE RECEPTORS IN CARP:

recognition of protozoan parasites



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This research was conducted under the auspices of the Graduate School of the Wageningen Institute of Animal Sciences.

Innate immune receptors in carp:

recognition of protozoan parasites

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Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr. M. J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday 1 October 2010
at 4 p.m. in the Aula

Innate immune receptors in carp:
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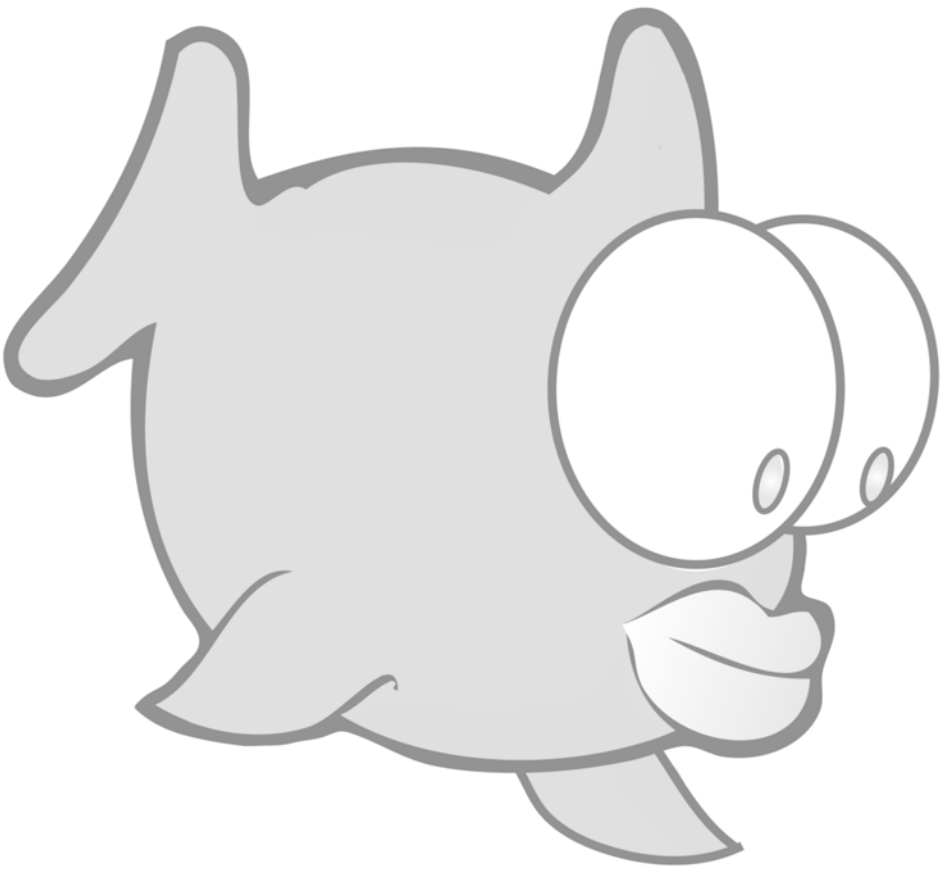
PhD thesis, Wageningen University, The Netherlands, 2010
With references, with summaries in English, Dutch and Portuguese

ISBN: 978-90-8585-774-7

Aos meus queridos
Ma, Pa e Mano

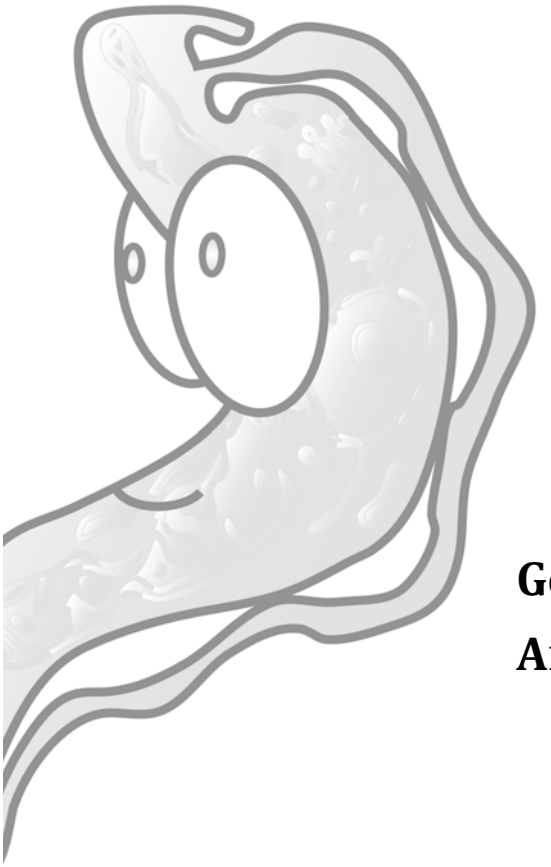
Contents



" The optimist says, The glass is half full
The pessismist says, The glass is half empty
The rationalist says, This glass is twice as big as it needs to be."

Thomas Cathcart & Daniel Klein
Plato and Platypus Walk into a Bar...

Chapter 1



General Introduction

Aims and Outline of the thesis

Innate immune receptors on macrophages

A fundamental interest in the evolution of immune mechanisms and, consequently, a detailed understanding of the fish immune system is essential for the correct implementation of prophylactic strategies, such as immunomodulation and vaccination, in aquaculture (1). Fish are the oldest vertebrate group in evolution that possesses all basic elements of the innate and adaptive immune system found in mammalian vertebrates (2). The important regulatory role for innate immune cells in recognizing pathogens and instructing adaptive immune reactions has led to a re-appreciation of the innate immune system. Central to innate immune responses are *macrophages* with a widespread tissue distribution and response to many different stimuli. Once a pathogen has breached physical barriers such as the skin or intestinal tract mucosa, it can be recognized by receptors on macrophages and other innate immune cells, triggering a whole series of immune responses aimed at the removal of the pathogen (3, 4). Macrophages can be activated after recognition of pathogens by *pattern recognition receptors* (PRRs). There are several distinct classes of PRRs, able to recognize a large array of *pathogen associated molecular patterns* (PAMPs) (5). These receptors are involved in performing tasks such as phagocytosis aimed at an adequate clearance of the pathogen by, for example, the production of oxygen and nitrogen radicals (6, 7). PRRs, therefore, play a central role in the detection of molecular structures unique to pathogens (providing what is called 'signal 0') and direct activation of innate host-defense mechanisms (Fig.1).

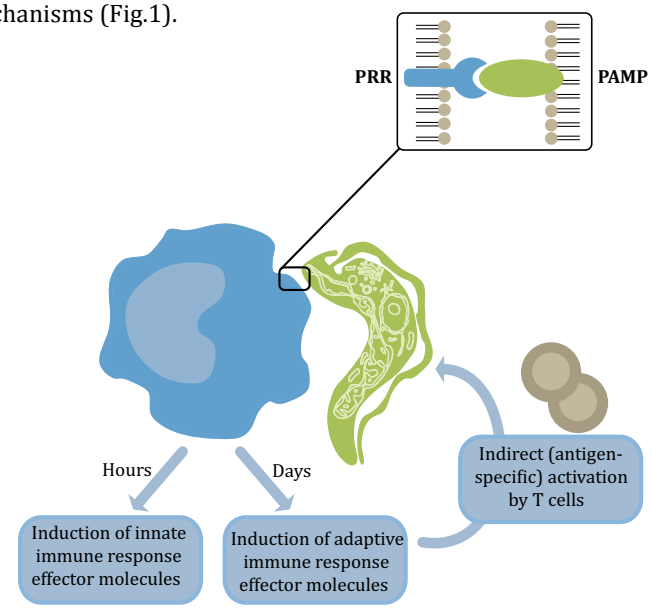


Figure 1. Activation of host defence mechanisms by engagement of pattern recognition receptors (PRRs). PRR engagement can directly induce host innate immune response effector molecules (e.g. radicals and antimicrobial peptides) and instruct the adaptive immune system to mount a response involving effector class molecules (e.g. cytokines and chemokines). After an adaptive immune response has been initiated, it can result in antigen-specific activation of innate immune cells by T cell-derived cytokines. Adapted from (6).

PRR engagement is also involved in the presentation of phagocytosed antigen to cells of the adaptive immune system (signal 1). The subsequent PRR-induced inflammatory cytokines can shape the adaptive immune system by downstream activation of distinct T helper cell subsets (referred to as signal 2) (8-10).

Teleost fish lack a bone marrow and lack lymph nodes. The *head kidney* (HK) exhibits morphological and functional similarities to the mammalian bone marrow and is considered one of the major haematopoietic organs in carp. Myelopoiesis generally occurs in the HK and fish macrophages derived from head kidney progenitors perform functions analogous to macrophages from mammalian vertebrates (e.g: phagocytosis, radical production and cytokine secretion). Melano-macrophage centres in HK and spleen have been suggested to play a role analogous to the germinal centres found in lymph nodes of mammalian vertebrates (11-13). Although the place where antigen presentation takes place in teleost fish is still under debate, there is no doubt about the presence of PRRs such as those belonging to the family of *Toll-like receptors* (TLRs) or receptors belonging to the *Ig superfamily* on teleost macrophages (14, 15).

Toll-like receptors (TLRs)

TLRs are evolutionary-conserved molecules which were originally identified in vertebrates on the basis of their homology with the protein coded by the *Toll* gene, first identified in *Drosophila* (16). Functional studies revealed that *Drosophila* Toll is required for disease resistance to fungal pathogens (17). TLRs are type-I transmembrane proteins with several extracellular *leucine-rich repeat* (LRR) motifs and an intracellular *Toll/interleukin-1 receptor* (TIR) domain (7, 18). Crystallization of the ectodomain of human TLR3 (19) and subsequent crystallization of the ectodomain of human TLR2 (20) revealed that the numerous LRR motifs in a TLR molecule together form a horseshoe-like shaped solenoid (Fig. 2) that is directly involved in ligand interaction. Ectodomains of different TLRs can interact with different ligands including lipids, carbohydrates, proteins and nucleic acids (5). LRR motifs have also been proven crucially important as recognition domains also for a number of cytosolic proteins such as nucleotide oligomerization domain (NOD) receptors and Nacht, LRR and Pyrin domain-containing proteins (NALP)(21).

The intracellular TIR domain is compact and globular and is also found in interleukin 1 and interleukin 18 receptors that do not have extracellular LRR but do have immunoglobulin-like molecules as recognition domains (22). In general, recognition of PAMPs by TLRs results in recruitment of a set of TIR domain-containing adaptor proteins such as MyD88. These interactions trigger intracellular downstream cascades eventually leading to mitogen-activated protein kinases (MAPKs), activation of nuclear factor-κB (NF-κB) and subsequent secretion of cytokines (23).

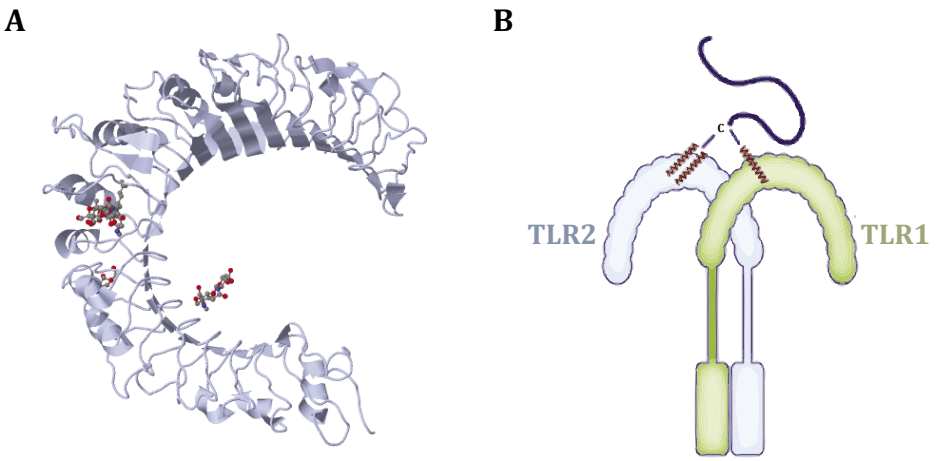


Figure 2. A) Extracellular domain of a Toll-like receptor (TLR) with numerous leucine-rich repeat (LRR) motifs together forming a horseshoe-like shaped structure (human TLR2-lipoteichoic acid complex, PDB no:3A7B). B) Heterodimerization of TLR2 with TLR1 and schematic presentation of triacylated ligand binding. Adapted from (24).

Analysis of genomes of vertebrates as distant as teleost fish and primates has revealed a minimal number of ten genes encoding different TLRs, which can be classified into six major families: TLR1, 3, 4, 5, 7 and TLR11 (25). In humans, TLR1 to 13 found on chromosomes 1, 3, 4, 9 and the X chromosome have been described (26). TLRs within a family share distinct sequence characteristics and recognize a general class of PAMPs. Most vertebrate genomes have at least one gene representing each of the six major TLR families (25). However, there are some exceptions to the rule. Fish species belonging to the Order Salmoniformes (e.g. rainbow trout and salmon), Perciformes (e.g. seabream and seabasss), Pleuronectiformes (e.g.: flounder) and Tetraodontiformes (e.g. pufferfish) all lack the TLR4 gene. Remarkably, fish species belonging to the Order Cypriniformes (e.g. carp and zebrafish) do have present in their genome a TLR4 gene (27, 28). All teleost fish seem to lack TLR6 whereas birds (e.g. chicken) lack TLR9. Furthermore, several animals possess TLRs not present in the genome of mammalian vertebrates. For example, additional TLRs have been described for fish (TLR18-23), birds (TLR 15, 21) and amphibians (TLR14, 16) (29).

Immunoglobulin superfamily (IgSF) receptors

Most innate immune receptors of the *immunoglobulin superfamily* (IgSF) are type I transmembrane proteins containing one or more extracellular Ig-like domains with a typical barrel-shaped structure (Fig. 3), a transmembrane domain and a cytoplasmic region that may contain activating or inhibiting signalling motifs (15, 30). Classical stimulatory receptors have a short cytoplasmic tail only but can contain a positively charged amino acid residue that allows association with ITAM (immune receptor tyrosine-based activation motifs)-containing transmembrane adaptor proteins (31). In contrast, classical inhibitory receptors can have long cytoplasmic tails with a variable number of ITIMs (immune receptor tyrosine-based inhibition motifs) (32). Several important IgSF receptors are clustered together in the leukocyte receptor cluster (LRC) on human chromosome 19 (33). In addition to this cluster, several multigene families exist, for example, the triggering receptors expressed on myeloid cells (TREMs) and the TREM-like transcripts (TLT) found on human chromosome 6 (34). More distant relatives are the CD300 family and the polymeric Ig receptor (pIgR) present on human chromosome 17 and 8, respectively (35).

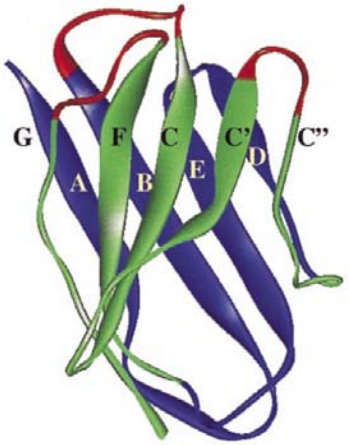


Figure 3. Schematic representation of a typical barrel-shape structure of an immunoglobulin (Ig)-like domain. The ABED β-sheet is coloured in blue and the GFCC'C'' β-sheet coloured in green. The three loops that make up the hypervariable regions interacting with the relevant antigenic epitope are indicated in red. Adapted from (36)

Novel immune-type receptors (NITRs) were found in a large number of teleost fish species (zebrafish, rainbow trout, gilthead seabream) and share structural and signaling similarities with mammalian vertebrate killer inhibitory receptors (KIRs), found in the human leukocyte receptor cluster (37, 38). Modular domain immune-type receptors (MDIRs) from cartilaginous fish (clearnose skate) share structural similarities with mammalian CD300, TREM/TLT and pIgR receptors, whereas novel immunoglobulin-like transcripts (NILTs) found in carp and rainbow trout share structural similarities with mammalian TREM and NKp44 receptors (39-41). The presence of distinct IgSF receptor families in non-mammalian vertebrates can reveal unique mechanisms of action present in teleost fish only.

Origin and evolution of TLR and IgSF receptor genes.

The origin of TLRs has been predicted to date more than 600 millions years ago (mya), before the separation of bilaterians and cnidarians (Hydra or coral) (29). However, the LRR motifs are even older and have been found in most disease resistance (R) proteins in plants (42). Conversely, TIR-containing receptors with short extracellular domains devoid of LRR motifs are found in most cnidarian species and sponges (43). The existence of these TLR-related genes suggests that the acquisition of extracellular LRR motifs occurred later during metazoan evolution (44). The fruitfly Drosophila has 9 TLRs members but *Toll*9 is the only member that groups with vertebrate TLRs (27, 45). Only a single TLR gene has been found in *Caenorhabditis elegans*, *tol-1*, whereas an expansion of TLRs was found in invertebrate deuterostomes, such as sea urchin (222 TLR genes) and in amphioxus (42 TLR genes) (46-48). No full genome sequence is available for jawless vertebrates, but recently two TLR14-like receptors have been identified in the lamprey (49). In addition, variable-lymphocyte receptors (VLRs), which are LRR-containing receptors that undergo somatic rearrangement for generation of immune receptor diversity, have been described in lamprey (50). PAMPS, recognized by vertebrate TLRs, are essential for microbial survival and by definition do not allow for rapid mutation, most probably contributing to the high conservation observed throughout vertebrate TLRs evolution. In conclusion, comparative sequence analysis reveals that vertebrate TLRs evolved at the same relatively slow rate, suggesting a strong selection to maintain their characteristic function (25, 29).

Immunoglobulin-domain containing proteins constitute the largest repertoire of surface receptors in animals and serve many functions in molecular recognition, cell adhesion and signaling (51, 52). Several different mechanisms have been described to create variation among IgSF receptors (53, 54). However, somatic rearrangement of highly diverse immune receptors has been considered to exist in a relatively small number of animal species restricted to the jawed vertebrates. Comparative studies revealed diversity of IgSF receptors also in Protostomes. Arthropods such as *Drosophila melanogaster* have the potential to express more than 18 000 isoforms of the IgSF receptor Down syndrome cell adhesion molecule (*Dscam*) (55). Somatic variation of soluble fibrinogen-related proteins (FREPs), with one or two amino N-terminal Ig-like domain and a C-terminal fibrinogen domain, was observed in mollusks (56). Among the invertebrate deuterostomes, amphioxus expresses the variable-region-containing chitin-binding proteins (VCBPs). VCBPs consist of two N-terminal V –type Ig domains and a C-terminal chitin-binding domain and exhibit extremely high levels of polymorphism (57). These observations, coupled with the wide range of multigenic IgSF receptors families (NITRs, KIRs, Ig) described in jawed vertebrates, suggest that IgSF domains are subject to a rapid rate of evolution with frequent emergence and extinction of gene families as they are selected for their utility in immune surveillance (15, 51).

Protozoan parasite recognition

Adaptation and evasion strategies have allowed the *Kinetoplastida* (e.g.: *Trypanosoma brucei*, *Trypanosoma cruzi*) to persist in almost all vertebrate groups and infections with these protozoan parasites are widespread not only among warm-blooded but also among cold-blooded vertebrates (58, 59). Several TLR agonists derived from protozoan parasites have been identified. There is evidence that recognition of DNA from *T. cruzi* and *T. brucei* is TLR9 mediated (60, 61). Furthermore, glycosylphosphatidylinositol (GPI) anchors from protozoan parasites as diverse as *T. cruzi* and *Toxoplasma gondii* can activate myeloid cells in a TLR2-dependent manner (62, 63).

The order Kinetoplastida diverged into the sub-orders Trypanosomatida and Parabodonida some 200–300 million years ago (64); both sub-orders include protozoan pathogens of common carp. *Trypanoplasma borreli* (Parabodonida) and *Trypanosoma carassii* (Trypanosomatida) both are extracellular parasites that live as extracellular forms in the bloodstream of carp and are transmitted by leech vectors. In nature, mixed infections of *Trypanoplasma borreli* and *Trypanosoma carassii* can be found (59, 65). In the laboratory, mostly mono-parasitic infections are studied. In this thesis, these two parasites were used as infection models to study innate recognition as part of the immune response of carp to infection.

Aims and outline of the thesis

The hypothesis pertinent to this thesis is that carp macrophages bear pattern recognition receptors essential to the recognition of the protozoan parasites *T. borreli* or *T. carassii* and central to the development of (protective) innate immune responses. Research on fish innate immune receptors can give insight into the mechanistic basis for the rational design of a vaccine based on PRR ligands and can shed light on the evolution of innate immune receptors involved in the recognition of pathogens.

Macrophages, activated after detection of unique molecular structures (signal 0), can present pathogen-derived antigen to cells of the adaptive immune system (signal 1) and secrete cytokines that shape the adaptive immune system (signal 2) leading to pathogen clearance. Both antigen presentation and additional secondary signals are required for activation of specific B and T lymphocytes. The vast majority of current vaccines act by inducing neutralizing antibodies, however, many new vaccine targets require the induction of specific cell-mediated responses. In general, adjuvants can be categorized as "signal 1 facilitators" and /or "signal 2 facilitators" according to their mode of action (**CHAPTER 2**). Improved knowledge on the modes of action of immunopotentiators can lead, also in fish, to a more efficient design of vaccines for aquaculture practice.

To study the functional consequences of innate immune receptor engagement, we first developed an *in vitro* culture system to obtain relatively pure cultures of carp macrophages derived from the head-kidney, the haematopoietic organ in carp (**CHAPTER 3**). These macrophages, best compared with mammalian bone-marrow derived macrophages, retain the ability to phagocytose, produce radicals and polarize into innate activated or alternatively activated macrophages. The characterization of this carp macrophage culture endows an important technological platform to subsequently study the fundamental immune mechanisms of macrophage innate immune receptors in comparative immunology. In the following chapters, we refer to these head-kidney derived macrophages as macrophages.

Details of how pathogens such as trypanosomes can activate pattern recognition receptors on (carp) macrophages are scarce because of limited knowledge on the ligand-receptor interactions in fish macrophages. In mammalian vertebrates, TLR2 is an important PRR that senses, among others, peptidoglycan from Gram-positive bacteria. In teleost fish, research on TLR2 had been mostly restricted to the sequence identification of TLR2 genes in a few fish species, with limited studies on induction of gene expression. Although these studies demonstrated the presence of a TLR2 orthologue in fish, the ligands for the fish TLR2 receptor and the TLR2-induced downstream signaling cascades has not been studied in detail. We transfected carp TLR2 in human embryonic kidney cells and overxpressed TLR2 in carp macrophages to study carp TLR2-mediated immune responses to ligands from Gram-positive bacteria (**CHAPTER 4**).

In mammalian vertebrates, TLR2 also senses glycosylphosphatidylinositol anchors from protozoan parasites. Live protozoan parasites *T. borreli* or *T. carassii* and GPI anchors derived from both protozoan parasites were examined as TLR2 ligand (**CHAPTER 5**).

The immune response of carp against these two parasites is fundamentally different. *T. borreli* infections are associated with classically-activated macrophages with a high production of TNF-α, IFN-γ and nitric oxide (NO). In contrast, *T. carassii* infections are associated with alternatively-activated macrophages, do not lead to NO production and these infections last for 2-3 weeks longer. The difference in host response to these two related parasites could be driven by the initial PRR engagement on carp macrophages. For example, recognition via mammalian TLR2 can lead to a preferential production of IL-23, promoting a Th17-mediated immune response. We examined the regulation of different IL-12 cytokine family members (p19, p35 and p40a-c) during infection of carp with *T. borreli* and *T. carassii* and studied the role of TLR2 in mediating the immune response against these two parasites.

In mammalian vertebrates, TLR9 is an important PRR that senses, among others, DNA-containing unmethylated CpG motifs found in bacterial and protozoan (trypanosome) DNA (**chapter 6**). Studies in teleost fish had demonstrated the presence of TLR9 orthologues and immunostimulatory activities for CpG oligodinucleotides (ODNs) had been reported for several fish species, but not in carp. In mammalian vertebrates, the subcellular localization and mobilization of TLR9 to DNA-containing endosomes promotes a proteolytic cleavage of TLR9 by resident proteases. We examined synthethic CpG ODNs and DNA from pathogens as putative ligands for carp TLR9 and studied the regulation of TLR9 activation in carp macrophages.

The choice to examine TLR2 and TLR9 function in carp is based on literature studies of parasite-host interactions and ligand recognition by macrophages of warm-blooded vertebrates. This candidate gene approach, however, does not take into account inherent differences between PRRs of warm- and cold-blooded vertebrates. In this thesis we also took an unbiased approach (**chapter 7**), identifying (novel) receptors encoded by carp macrophages and regulated by protozoan fish parasites. We generated, by suppression subtractive hybridization (SSH), a subtracted cDNA repertoire from carp macrophages enriched for genes up-regulated in response to the protozoan parasite *T. borreli*. We identified and characterized a novel soluble immunoglobulin-like receptor in carp which we named Soluble Immune-Type Receptor (SITR) and analysed the zebrafish genome for SITR orthologues. The relation of fish SITRs to other IgSF receptors described for fish (e.g. NITRs) and mammals (e.g. CD300) will be discussed. We applied a reverse genetic approach using morpholino antisense technology in carp macrophages and overexpression in mouse RAW macrophages to study the involvement of carp SITR in *T. borreli*-induced immune responses.

In the last chapter of this thesis (**chapter 8**), we discuss the importance of our findings on innate immune receptors for the immune response of carp to protozoan blood parasites and discuss the evolution of innate immune receptors involved in the recognition of pathogens.

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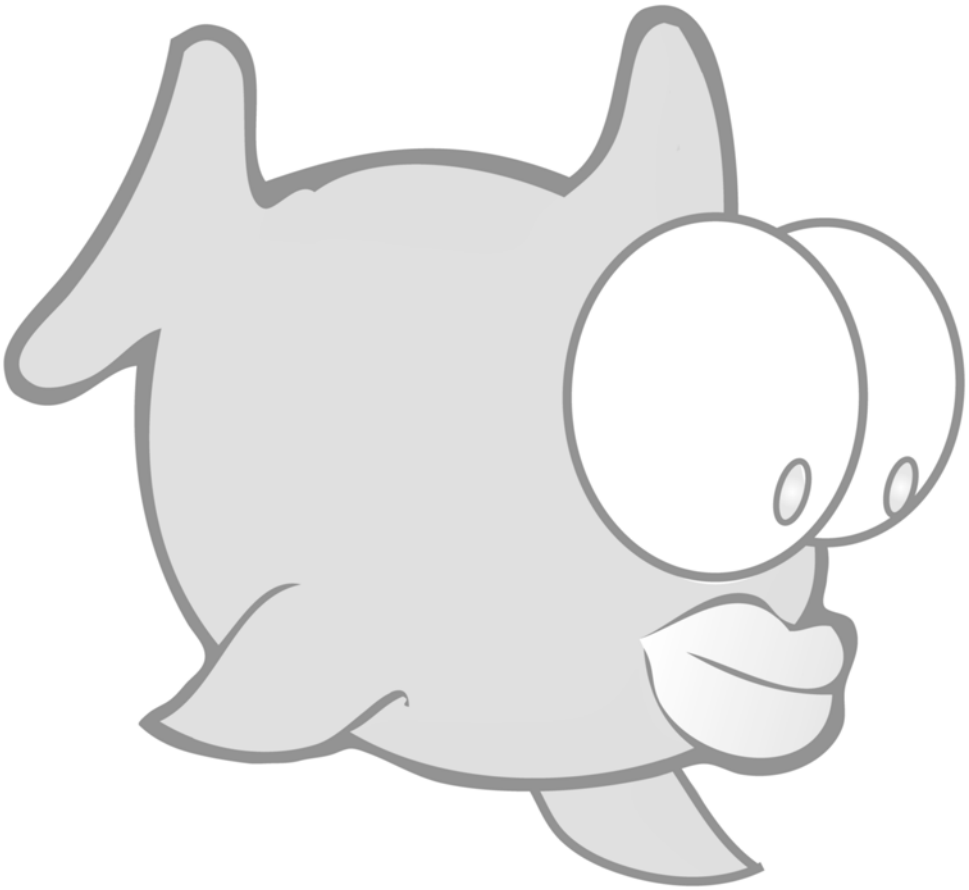
" A person who never made a mistake never tried anything new. "

Albert Einstein



Chapter 2

Immunology of Vaccine Adjuvants

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Vaccine Adjuvants, Methods in Molecular Biology, 2010
(626), 1-14 Gary Davies (Editor), Humana Press Inc.

ABSTRACT

In recent times vaccine adjuvants, or immunopotentiators, received abundant attention in the media as critical ingredients of current and future vaccines. Indeed, vaccine adjuvants are recognized to make the difference between competing vaccines based on identical antigens. Moreover, it is recognized that vaccines designed for certain indications require a matching combination of selected antigen(s) together with a critical immunopotentiator that selectively drives the required immune pathway with minimal adverse reactions. Recently, the mechanistic actions of some immunopotentiators have become clearer as a result of research focused on innate immunity receptors. These insights enable more rational adjuvant and vaccine design, which, ideally, is based on predictable immunophenotypes following vaccination. This chapter addresses immunopotentiators, classed according to their (presumed) mechanisms of action. They are categorized functionally in two major groups as facilitators of signal 1 and/ or signal 2. The mode(s) of action of some well-known adjuvant prototypes is discussed in the context of this classification.

INTRODUCTION

Vaccines have become one of most successful life-saving instruments in modern medicine due to their extremely efficient and cost-effective prevention of infectious disease (1). Traditionally, vaccines comprise either live attenuated, replicating pathogens or non-replicating, inactivated pathogens or their subunits (2). Live vaccines, still used to immunize against measles or rubella, are safe for the majority of recipients. Although cost-effective and rather easy to manufacture, live vaccines may cause disease when given to a recipient with an unrecognized immunodeficiency (3). Inactivated vaccines consist of killed pathogens or isolated non-replicating subunits. They are safe for immunocompromised individuals, but they often show limited immunogenicity. The vast majority of current vaccines act by inducing antibodies. However, many new vaccine targets require the induction of specific cell-mediated responses, in addition to antibodies (4). Special adjuvants, or immunopotentiators, are therefore required to elicit adequate immunity, for example, enhancement of T-cell responses, by targeting certain innate immune cells in most cases, with the additional benefits that less antigen doses and fewer administrations are necessary (5). Vaccine adjuvants come in many forms and are more or less effective at inducing the onset, magnitude, duration and quality of an immune response against a co-formulated antigen. Hence, adjuvants (from Latin *adjuvare* meaning "to help") can be defined as a group of structurally heterogeneous compounds able to enhance or modulate the intrinsic immunogenicity of an antigen (6). They can be classed according to their chemical nature or physical properties, yet related compounds frequently have divergent immunomodulating capacities. For example, saponin variants may differ in their

capacity to stimulate Th1 or Th2 type immunity (7). Alternatively, adjuvants have been clustered according to the immunological events they induce (2, 8), although for many the exact mechanism of action is unknown. In 1989 Charles Janeway Jr. aptly termed vaccine adjuvants "the immunologist's dirty little secret" (9). It reflected the general ignorance on the mechanisms of action of most known adjuvants at the time. Yet rational vaccine design involves a logical choice of immunopotentiator based on its mode of action and its expected effect on efficacy and safety of the vaccine (3). Despite the tremendous impact of the adjuvant choice, even today the key processes critical for immune induction in general, and those evoked by distinct adjuvants, in particular, are unknown and subject of debate among immunologists and vaccinologists (10).

SIGNAL 1 AND SIGNAL 2 FACILITATORS

At present two major functional classes of vaccine adjuvants have been defined (8). The first category includes so-called facilitators of signal 1, influencing the fate of the vaccine antigen in time, place and concentration, ultimately improving immunoavailability of the antigen. The second major group constitutes facilitators of signal 2, providing the correct co-stimulation signals for the antigen-specific adaptive immune cells during antigen recognition. Both classes of adjuvant are not mutually exclusive (Table 1) For a schematic illustration *see* Fig.1.

Table 1: Classification of adjuvants according to their stimulatory action

Adjuvant category	Concept	Critical feature	Example
Signal 1	Improving immunoavailability	Time, place, dose of antigen	Alum-containing adjuvants, oil-based emulsions
Signal 2	Improving co-stimulation	Stranger (PAMP)	Lipopolysaccharide
		Danger (DAMP)	Heat-shock proteins
		Recombinant co-stimulus	Recombinant interferon
		Release of natural immune system brakes	CTLA-4 inhibitory antibody

According to the two-signal model (11, 12) both the presentation of antigen (signal 1) and additional secondary signals (signal 2) are required for activation of specific T and B lymphocytes, which form the adaptive arm of the immune system. Secondary signals are delivered by co-stimulatory or co-inhibitory signals, and their overall balance and constellation determines the magnitude and quality of the ensuing adaptive immune reaction (Fig. 1). Stimulatory second signals (in most cases for adjuvants) inform the T cells that the presented antigens are proper subjects to initiate an immune response.

Charles Janeway predicted that microbial pathogens are evolutionarily distant from their hosts and contain conserved molecular patterns, so-called pathogen-associated

microbial patterns (PAMPs), which can be recognized by so-called pathogen recognition receptors (PRRs) (9). Today we know that PRRs exist. They include Toll-like receptors (TLRs), NOD-like receptors, dectin-1, or RIG-like helicases which are predominantly found on cells of the innate immune system. The rather recent discovery of PRRs and their capacity to detect molecular structures unique to pathogens (also referred to as signal 0) provided a mechanistic concept of the key upstream events leading to co-stimulation. This insight formed a breakthrough for rational vaccine design based on PRR ligands, comprising various PAMPS and later also endogenous TLR ligands, such heat shock proteins (Hsp) (5). Nowadays the innate immune system is studied extensively after the general appreciation by the scientific community that this system plays a critical role in PAMP sensing and instruction of adaptive immune reactions. It is considered critical in signal 2 induction and downstream activation of distinct T helper cell subsets. The category of signal 2 facilitating adjuvants has since been mechanistically defined at the molecular level (please see further the section on signal 2 facilitators).

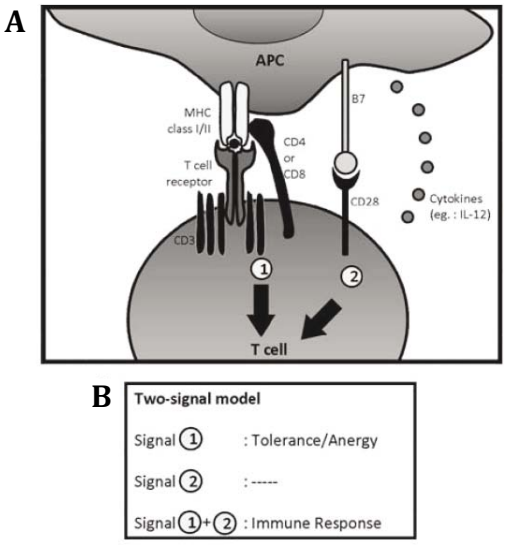

Figure 1: The two-signal model. Recent advances in immunology have shown that the magnitude and specificity of the signals perceived by the innate immune cells following infection (and vaccination) can shape subsequent adaptive immune responses. Activation of (depicted) T helper (Th)-cells requires at least two different signals from the antigen-presenting cell (APC) including signal 1 (antigen presentation) and signal 2 (co-stimulation).

SIGNAL 1 FACILITATORS

In contrast to molecular events involved in the generation of signal 2, very little is known about the mechanisms governing the adjuvant effect of signal 1 facilitators. When soluble antigen is injected subcutaneously it is presented in two waves in the secondary draining

lymph nodes (LN). Within 30 minutes free antigen enters the LN by afferent lymph vessels and is presented by resident dendritic cells (DC), while tissue resident DCs that acquire antigen at the injection site migrate to LN within 12-24 hours and present Ag in MHC class II complexes. They sustain the activation of Ag-specific $CD4^+$ cells initially activated by resident DC. (13).

For most vaccine adjuvants it is not known whether their activity is required at the site of injection or in the local draining lymph node. Naive T and B cells normally do not enter non-lymphoid areas, such as most injection sites, of the body efficiently. They rather circulate between secondary lymphoid organs, including the spleen and lymph nodes, via the blood and efferent lymphatics. Only memory and effector lymphocytes are able to penetrate non-lymphoid tissues during an inflammation (14, 15). Hence the antigen has to reach the secondary lymphoid tissues in order to be recognized by adaptive immune cells.

The geographical concept of immune reactivity (16) proposed that time, place and dose of the antigen are critical factors for induction of adaptive T and B cell responses. It is supported by experimental observations of reduced immune responses early after surgical removal of the injection site (17) and in lymph node deficient hosts (18). No immune response develops when the antigen is not able to reach the lymph node due to interruption of the afferent lymphatics (19, 20, 21, 22). Also the fact that augmented immune responses were observed after repeated injection of minute amounts of poorly immunogenic antigens support this concept (23, 24). Obviously, motility and migration have a key role in many biological processes.

However, despite the paramount importance of this class of vaccine adjuvants and their extensive application in existing vaccines, the mechanisms underlying the activity of presumed signal 1 facilitators remain a mystery. For prototype examples such as oil-based emulsions and aluminium-based adjuvants some aspects of its alchemy are described in the next section.

Oil-based emulsions

Already in 1968 Herbert attempted to mimic slow release of antigen by daily injections of tiny doses of ovalbumin in mice, and noted an antibody production profile similar to a single full-dose of ovalbumin formulated in a W/O emulsion (24). Interestingly, the antibody level in the circulation dropped soon after the daily injections were stopped.

Freund studied the role of the depot function in rabbits by measuring antibody formation after surgical removal of the vaccine from the site of injection. Excisions performed between 30 minutes and four hours after injection resulted in a decrease of the immune response when compared to the response in animals in which the injected areas were not removed. Remarkably, excisions performed after one or more days did not seem to affect the antibody response (17, 25). Hence, as yet there is no conclusive evidence for slow (i.e. more than one day) release as explanation for immune stimulating features of Freund's adjuvants. Despite such investigations, little is known about the mechanisms responsible for adjuvant activity of the W/O emulsion in general. The structural requirements as well as the cellular and molecular immunological

mechanisms within the host remain poorly understood. Indeed, this extremely powerful immune activating formulation has been shrouded in obscurity and is often referred as an example of "the immunologist's dirty little secret". As local reactions and residues represent major safety concerns for vaccines based on W/O emulsions, there is a clear demand for alternatives. Understanding the mechanism underlying W/O emulsion activity may help to further improve their formulation, thereby diminishing or eliminating potential hazards. Alternatively, such insights will be highly useful in developing novel vehicles in general.

Dupuis and coworkers (26) showed that DCs internalize vaccine antigen and labelled adjuvant MF-59, an oil-in-water emulsion, after intramuscular injection. How DCs know where to go is likely based on chemokine-based attraction, preceded by recruitment of granulocytes and monocytes/ macrophages as shown recently (27). In general, these cells are rapidly recruited into sites of tissue injury in response to inoculation with live or inactivated microorganisms, probably as a result of locally produced chemotactic factors. The transient influx of neutrophils (PMNs) or other innate immune cells (27) likely affects DC function. Upon arrival in the lymph nodes DC's antigen capture and processing capacity declines, while their immunostimulatory function is upregulated.

Aluminium-based adjuvants

Aluminum-containing adjuvants such as aluminium hydroxide and aluminum phosphate adjuvants (often referred to as alum) are generally assumed to adsorb the Ag on the alum particle which forms an Ag depot at the injection site. This is believed to prolong availabililty of Ag (signal 1) for Ag presenting cells (APCs). However, this concept has been challenged in recent times. various mechanisms have been proposed to be responsible for its adjuvant effect. Alum has been shown to fix complement (28), to cause granulomas (29), to recruit eosinophils and neutrophils (30) and induce the appearance in the spleen of IL-4-secreting cells (31). Yet, how alum achieves these effects remains unknown.

Interestingly, alum does not promote classical direct dendritic cell (DC) maturation (32), which questions whether its activity is mediated by pattern recognition receptors. Kool and coworkers showed that intraperitoneally injected alum triggers local inflammatory $CD11b^{+}Ly6G^{-}Ly6C^{+}F4/80^{int}$ monocyte type cells to differentiate into inflammatory dendritic cells, which is associated with the secretion of uric acid (33). Treatment with uricase reduced recruitment of antigen (OVA)-labelled monocytes in alum immunized mice. However, effects on alum-driven antigen-specific antibody production were not measured. This concept is strongly challenged by earlier data showing that uric acid crystals augment CTL responses to co-injected antigen, which, by contrast, was not observed for aluminium hydroxide in the same animal experiments (34). Hence, uric acid polarizes the immune response towards a type-1 response associated CTL reaction, while a Th2 type response is the hallmark of alum adjuvanted vaccines. The lack of proper CTL priming by alum represents its major deficiency as vaccine adjuvant.

Both (35), and very recently (30) showed that alum is able to activate the NALP3

inflammasome in human peripheral blood mononuclear cells (PBMCs), and primary peritoneal macrophages of mice, respectively. However, such activation is dependent on priming of the cells with lipopolysaccharide (LPS), questioning whether inflammasome activation is involved during vaccination in the absence of a costimulatory LPS signal. By contrast, Sokoloska and co-workers showed in 2007 that alum-containing adjuvants directly stimulate the release of IL-1β and IL-18 via caspase-1 activation from mouse dendrictic cells. Remarkably, in this study responses were noted without priming by LPS (36). In NALP3-deficient mice a partial (3 to 5-fold) reduction of alum-induced antigen-specific antibody formation (IgE and IgG1 isotypes) was observed using OVA or Human serum albumin (HAS) as antigens (30, 35). On the contrary, Franchi and Nunez showed that Nlp3 deficient mice showed normal antibody responses when immunized in the context of alum adjuvant (37).

However, of particular importance is a recent study of (38) demonstrating that mice lacking MyD88, and therefore unable to respond to TLR signals as well as IL-1β and IL-18 (39), are still capable to generate normal antibody responses when augmented by repository vaccine adjuvants including aluminium hydroxide, Freund's incomplete and complete adjuvant. Of importance is also the study of Pollock et al., who showed that endogenous IL-18 facilitates alum-induced IL-4 production, but effects on humoral immune responses such as OVA-specific immunoglobulin G1 (IgG1) and IgE production, the hallmark of alum's adjuvant effects, remained unaffected in IL-18 deficient mice (40). Moreover, in an allergic asthma model, IL-1R1-deficient mice sensitized by OVA formulated in alum, developed typical pulmonary Th2 responses, eosinophilic inflammation, antibody responses, and CD4(+) T cell priming in lymph nodes similar to normal mice (41).

Together, these data do not support the recently proposed NALP3 pathway (30), nor the uric acid concept (33) for alum adjuvant activity. In addition, there is no evidence for IL-18 or IL-1β dependence.

Signal 2 facilitators

Signal zero, PAMPs, and stranger motifs

The innate immune system of vertebrates uses germline encoded pattern recognition receptors (PRRs) to sense invading pathogens. These PRRs are able to recognize so-called pathogen associated molecular patterns (PAMPs), for example, peptidoglycan or unmethylated CpG DNA. PAMPs are unique and conserved molecular structures of a given microbial class (bacteria, viruses, fungi and protozoa). Depending on their location these PRRs can be: *secreted receptors* (e.g.: pentraxins) found in blood and lymph associated with complement or opsonization; membrane receptors (e.g.: C-type like receptors, Toll like receptors) on APC associated with endocytosis or induction of nuclear factor-κB (NF-kB)- and mitogen-activated protein kinases (MAPKs)-dependent signaling pathways or cytosolic receptors on APCs associated with induction of NF-KB and MAPK signaling pathways (42) (Fig. 2).

Among the PRRs, Toll like receptors (TLRs) are the most well-studied and best-described class of receptors. They have been shown to play an essential role in the initial response of the innate immune system to infection. TLRs are type I transmembrane proteins with an extrcellular domain of interspersed leucine-rich repeat (LRR) motifs that are involved in recognition of PAMPs. The cytoplasmic domain is characterized by a Toll/IL-1 receptor (TIR) motif which is involved in signal transduction (43). TLRs are either expressed on the plasma or endosomal membrane of APCs and they respond to specific bacterial, viral, fungal and protozoan PAMPs. Recognition of PAMPs by TLRs results in recruitment of a set of TIR domain-containing adaptor proteins such as MyD88. These interactions trigger intracellular downstream cascades eventually leading to activation of nuclear factor-kB (NF-kB) and mitogen-activated protein kinases (MAPKs), which results in induction of inflammatory cytokines (e.g.: Interleukin-1β, Tumor necrosis factor-α) and co-stimulatory ligands (B7-1 and B7-2). Importantly, TLRs not only trigger signal 2. They may also play a role in antigen presentation (signal 1). TLRs may control the generation of T cell receptor (TCR) ligands from the phagosome guaranteeing the presentation of both microbial components and antigen to activated APCs (44). TLR engagement can initiate and also shape the adaptive immune response, for instance, by skewing the immune system towards a Th1 or Th2 response (45) or by activating or inhibiting Treg cells (46). Moreover, vaccine adjuvants that contain TLR ligands can induce higher avidity T cell responses (47). This suggest the need for antigen and TLR-agonist to be co-delivered, in order to target the same phagosome cargo of one APC and thereby induce optimal antigen presentation and subsequent stimulation of Ag-specific T- cell responses (6). Indeed, TLRs ability to link innate and adaptive immunity represents a promising mechanism to be explored in the design of new vaccines (5).

However, antigen targeting and engulfment can also be mediated by other receptors present in the surface of APC, such as C-type lectin receptors (CLRs) and triggering receptors expressed on myeloid cells (TREMs). CLRs, including mannose receptor and DC-SIGN recognize sugar moieties (e.g.: N-aceylglucosamine, mannose) at the surface of the pathogens enabling binding to an array of bacteria, virus and fungi. Intracellular cytosolic receptors such as NODs (nucleotide-binding oligomerization domain proteins), NOD-like receptors (NLRs) and retinoic acid-inducible gene I-like helicases (RLHs) recognize structures from intracellular bacteria or viruses, but possibly also aluminium-containing adjuvants (30) as mentioned earlier. These receptors may form new targets for vaccine adjuvant development.

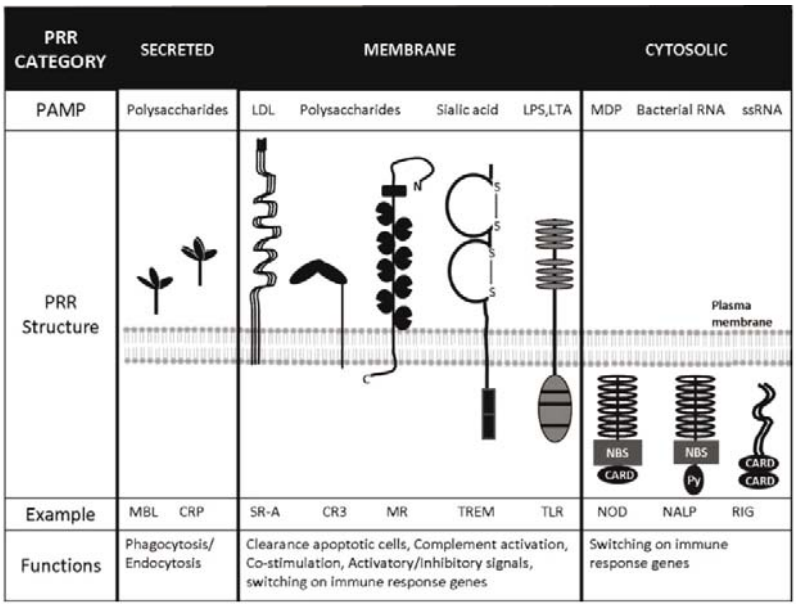

Figure 2: Overview of major classes of Pattern Recognition Receptors. MBL, Mannose binding lectin; CRP, C-reactive protein; SR, Scavenger Receptor; CR, Complement Receptor; MR, Mannose Receptor; TREM, Triggering receptor expressed on myeloid cells; TLR, Toll like receptor; NOD, Nucleotide oligomerization domain; NALP, Nacht, LRR, and PYRIN-domain containing proteins; RIG, Retinoic acid-inducible gene; LDL, Low density protein, LPS, Lipopolysaccharide, LTA, Lipoteichoic acid, MDP, muramyl dipeptide; ss, single stranded; NBS, Nucleotide binding site; CARD, caspase recruitment domain.

Danger signals, alarm signals or damage-associated molecular patterns (DAMP)s

According to the so-called danger theory, originally proposed by (48) the immune system evolved to focus primarily on danger rather than on microbial non-self signals described above (48, 49, 50) Accordingly, antigens can be divided in two major groups: those associated with danger or antigens positioned in a harmless situation. Danger signals can be released by stressed, or damaged tissue, as organelles of cells undergoing necrotic (but not apoptotic) death. Although such organelles or molecules from dead cells are not well defined at the molecular level, likely candidates responsible for observed immune activation include mitochondrial and nuclear fractions of necrotic cells as well as heat shock proteins (HSPs), cellular DNA or uric acid (30). These may be referred to as danger associated molecular patterns (DAMPs) which lead directly or indirectly to up-regulation of co-stimulatory signals for APCs. Together with antigens released from dying cells these danger signals are recognized by APCs which may trigger an immune response if the host is not tolerant for these antigens. Also endogenous cytokines, such as type IFN produced by infected cells, can be considered a DAMP. Indeed, several adjuvants are known to cause local damage at the injection site and may act by induction of danger signals. Examples

include aluminum hydroxide (30), saponins, and possibly also oil-based emulsions (51). Theoretically, the danger model may explain, in part, some of the mystery of signal 1 facilitating adjuvants described earlier.

Recombinant cytokines or co-stimulatory molecules mimicking endogenous immune amplifiers

Protection conferred by some vaccines, such as influenza subunit vaccine, is mainly achieved by induction of a humoral response. A humoral response is mediated by antibodies and their effector functions, which include neutralization and opsonization of pathogen and activation of the complement cascade. Nevertheless, to ensure that the pathogen is effectively eradicated, it is not only important to attain an adequate amount of antibody but also to generate a specific immunoglobulin isotype. Antibody isotypes differ in their ability to activate the complement cascade or the binding to receptors on phagocytes. Therefore, it is of great importance to use adjuvants which promote the synthesis of specific antibody isotypes which confer more protective activity (52).

Synthesis of particular antibody isotypes is strongly influenced by the combination of locally produced cytokines. For example, interleukin 5 (IL-5) or transforming growth factor β can augment IgA antibody formation, while interleukin 4 (IL-4) and interleukin 13 (IL-13) are able to induce switching towards the IgE isotype, and interferon-γ (IFN-γ) increases the synthesis of IgG2a antibody (53). A number of cytokines represent the hallmarks of a polarized immune reaction. For example IFN-γ and IL-12 are associated with Th1 type immune reactions, while IL-4, IL-5 and IL-13 are related to Th2 type immune pathways. Use of such molecules in recombinant form may skew responses in the desired direction. Therefore, recombinant cytokines constitute serious adjuvant candidates that are especially suitable for subunit vaccines, which are poorly immunogenic when compared to whole killed or live-attenuated pathogens (54).

In addition, ligands and receptors of co-stimulatory pathways represent an attractive target for the development of adjuvants. For instance, Type I IFNs promote DC maturation by increasing expression of co-stimulatory molecules including CD40, CD80 and CD86 and major histocompatability complex (MHC) antigen. Recombinant IFNs co-delivered with influenza vaccine have been shown to enhance protection against virus challenge (55). Also CD40 stimulation by agonistic antibodies exerts adjuvant effects (56). As a result, various co-stimulatory agonists are currently considered as an important new class of adjuvants (57).

Release of the brakes

As mentioned earlier TLR agonists can constitute potent adjuvants for infectious diseases. However, it has been demonstrated that certain TLR agonists were able to promote the induction of IL10-secreting Treg cells (58.) A recent study revealed that TLR agonists can induce IL-12 and IL-10 production concomitantly and therefore promote the development of Th1 and importantly also T reg cells (59). So during a physiological immune response stimulating signals are accompanied by natural inhibitory signals. It is now recognized

that CTLA-4 blockade (60), or depletion of Treg cell development enhances the efficacy of therapeutic vaccination against tumors (61). Also, selective inhibitors of MAPK-p38 in dendritic cell vaccines suppressed IL-10 and enhanced IL-12 production thereby augmenting Th1 response and suppressing T reg cells (59). Hence, attenuation of regulatory T cell induction by proper TLR agonist, or inhibition of natural immune response attenuating signals, may improve the efficacy of some vaccines, and represents a variant of signal 2 facilitation (59).

OUTLOOK

Despite accumulated knowledge on the adjuvant mechanisms of signal 2 facilitators described above, we know very little about the classical adjuvants, which are presumed to facilitate signal 1. In particular we lack knowledge on the most up-stream events, the earliest interactions between adjuvant and the tissue at the injection site. Future studies will certainly unravel these events and contribute to rational vaccine design.

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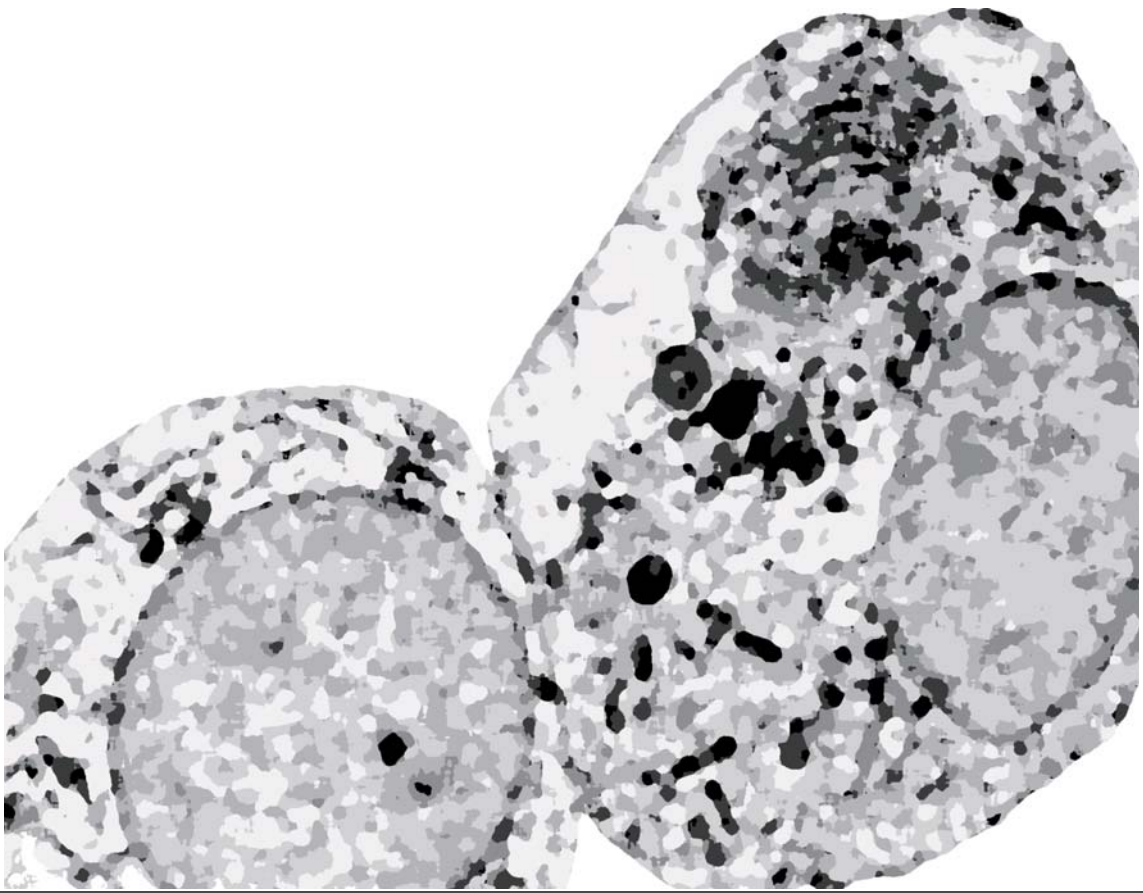
" The problem is that the right doesn't need any ideas to govern, but the left can't govern without ideas. "

José Saramago



Chapter 3

Head kidney-derived macrophages of common carp (*Cyprinus carpio* L.) show plasticity and functional polarization upon differential stimulation

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Journal of Immunology, 2006 (177), 61-69

ABSTRACT

Cells from the myeloid lineage are pluripotent. To investigate the potential of myeloid cell polarization in a primitive vertebrate species, we phenotypically and functionally characterized myeloid cells of common carp (*Cyprinus carpio* L.) during culture. Flow cytometric analysis, antibody labeling of cell surface markers and light microscopy showed the presence of a major population of heterogeneous macrophages after culture. These head kidney-derived macrophages can be considered the fish equivalent of bone marrow-derived macrophages and show the ability to phagocytose, produce radicals and polarize into innate activated or alternatively activated macrophages. Macrophage polarization was based on differential activity of iNOS and arginase for innate and alternative activation, respectively. Correspondingly, gene expression profiling after stimulation with LPS or cAMP showed differential expression for most of the immune genes presently described for carp. The recently described novel immunoglobulin-like transcript 1 (NILT1) and the CXCR1 and CXCR2 chemokine receptors were upregulated after stimulation with cAMP, an inducer of alternative activation in carp macrophages. Upregulation of NILT1 was also seen during the later phase of a *Trypanosoma carassii* infection, where macrophages are primarily alternatively activated. NILT1 could however not be upregulated during a *Trypanoplasma borreli* infection, a model for innate activation. Our data suggest that NILT1, CXCR1 and CXCR2 could be considered markers for alternatively activated macrophages in fish.

INTRODUCTION

Macrophages play a role in both the innate and the adaptive immune system. In the innate immune system they act as phagocytic cells, phagocytosing pathogens and producing oxygen and nitrogen radicals. In the adaptive immune system macrophages act as professional antigen presenting cells. As such, macrophages can provide a bridge between the innate and adaptive immune response. Altogether, macrophage differentiation exhibits a wide array of functional and phenotypic heterogeneity (1). Within this functional heterogeneity, classically and alternatively activated macrophages (caMF and aaMF, respectively) are proposed to represent the extremes of a continuum (2-4). In literature a further distinction has been made between classical and innate activation of macrophages (2). Classical activation is induced by stimulation with both IFNγ and a microbial trigger such as LPS, while innate activation is induced by stimulation with LPS (or other microbial triggers) alone (2). Although classically and innate activated macrophages are induced by different stimuli, their functions overlap. Innate activated macrophages have microbicidal activity and produce pro-inflammatory cytokines, ROS and NO (5). caMF find their role in type I immune responses against intracellular pathogens by the production of ROS and NO.

aaMF are active in type II immune responses against extracellular pathogens by showing increased phagocytic activity and enhanced gene expression of MHC class II. Furthermore aaMF increase their production of factors involved in tissue remodeling and repair and are able to inhibit type I inflammations (6). Macrophage activation in fish has been well studied with regard to NO production (7-11) and reactive oxygen species (ROS) (12-15). Recently, we described carp arginase gene expression and activity, which can be used as markers for alternatively activated macrophages, and we proposed an evolutionary conservation of alternatively activated macrophages down to teleost fish (16).

The aim of the present study was to investigate the macrophage polarization in teleosts, using functional assays and gene expression profiling. Hence we developed a primary cell culture system of carp head kidney-derived macrophages. In fish, only few such culture models exist for goldfish, rainbow trout and brook trout (17-19). We studied morphological changes by flow cytometry and light microscopy. We investigated functional changes by determining phagocytic ability, the ability to produce radicals (both ROS and NO) and by measuring arginase activity. A well-accepted way to study polarization is by determining gene expression profiles after differential stimulation. We quantified gene expression, by real-time quantitative PCR, in head kidney-derived macrophages stimulated with LPS or cAMP. Gene expression of carp CXCR1 and CXCR2 were upregulated after cAMP stimulation.

In addition to the CXCR1 and 2, head kidney-derived macrophages expressed the recently identified novel immunoglobulin-like transcripts (NILT)-1 and NILT2 (20) at different levels following stimulation. NILT1 and NILT2 are polymorphic receptors belonging to the immunoglobulin superfamily (IgSF) which, in general, recognize pathogen-associated molecular patterns. These type of receptors frequently exist in pairs with antagonistic signaling functions, are co-expressed on the same cell and bind similar, if not identical, ligands (21). The cytoplasmic region of NILT1 contains a cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM) and of NILT2 an immunoreceptor tyrosine-based inhibition motif (ITIM). The use of NILT1, CXCR1 and CXCR2 as possible surface markers for alternatively activated macrophages in fish is discussed.

Materials and Methods

Animals

Common carp (*Cyprinus carpio* L) were reared in the central fish facility 'De Haar-Vissen' at 23ºC in recirculating UV-treated tap water and fed pelleted dry food (Trouvit, Nutreco, France) daily. R3 x R8 heterozygous carp are the offspring of a cross between fish of Hungarian origin (R8 strain) and of Polish origin (R3 strain) (22). Carp were between 9 and 11 months old. All studies were performed with approval from the animal experimental committee of Wageningen University.

Cell culture for macrophages

Carp head kidney leukocytes (HKL) were cultured essentially as described before for goldfish and trout (17-19). Briefly, fish were euthanized with 0.25 g/l tricaine methane sulfonate (Crescent Research Chemicals, Phoenix, USA) buffered with 0.38 g/l $NaHCO_3$, bled by syringe from the caudal vein and head kidneys were aseptically removed. Head kidneys were gently passed through a 100-µm sterile nylon mesh and rinsed with homogenization buffer [incomplete-NMGFL-15 medium containing 50 U/ml penicillin, 50 µg/ml streptomycin, and 20 U/ml heparin (Leo Pharma BV, Breda, Netherlands)] (17). Cell suspensions were layered on 51% (1.071 g cm^{-3}) Percoll (Amersham Pharmacia Biotech AB, Uppsala, Sweden) and centrifuged at 450 x *g* for 25 min at 4°C without brakes. Cells at the medium/Percoll interface were removed and washed twice. Cell cultures were initiated by seeding $1.75x10^7$ HKL in a 75 cm^2 culture flask containing 20 ml of complete-NMGFL-15 medium [incomplete-NMGFL-15 medium supplemented with 5% heat-inactivated pooled carp serum and 10% bovine calf serum (Invitrogen, Breda, The Netherlands)] with 50 U/ml of penicillin and 50 µg/ml streptomycin. Cells were incubated at 27°C and head kidney-derived macrophages were harvested after 6 days by placing the flasks on ice for 10 min and gentle scraping.

Flow cytometry

Flow cytometry was used to follow the development of the cultures, forward scatter (FSC-H, reflecting cell size) and sideward scatter characteristics (SSC-H, reflecting internal cell complexity) were recorded for 10^4 events per sample, using a flow cytometer (Beckman Coulter, Epics XL-MCL, Miami, Florida USA). For all cytometric measurements the same settings were used: FS 350 volt, gain 2; SS 700 volt, gain 10; FL-1 800 volt, gain 1; FL-2 870 volt, gain 1; FL-3 675 volt, gain 1 and FL-4 950 volt, gain 1. The baseline offset was on and the discriminator at the FS at 20. For the phagocytosis assay a gate was set to measure cells only and not the (smaller) bacteria. Propidium iodide (PI 0.1 µg/ml) was added to each sample to detect and gate out PI^+ cells, and 100 µl of a standard diluted bead solution (Fluoresbrite® YG Carboxylate Microspheres 10 µm, Polysciences Inc, Warrington) was added to determine the amount of cells in culture. Cell populations were identified by FSC-H/SSC-H as described by van Kemenade *et al.* (23) for freshly isolated head kidney leukocytes (HKL) and by MacKenzie *et al*. (19) for head kidney-derived macrophages.

Cell surface markers (WCI12, TCL-BE8 and WCL15)

To further characterize the cell culture we determined the number of WCI12, TCL-BE8 and WCL15 positive cells at day 0, 2, 4 and 6 of culture. WCI12 binds to carp Ig, staining Ig-immunopositive B lymphocytes (24). TCL-BE8 mainly binds to neutrophilic granulocytes but also has cross reactivity with monocytes (25). WCL15 has been shown to bind to macrophages and monocytes and to a lesser extend to basophilic granulocytes in head kidney cell suspensions (26). Cells ($2.5x10^5$ per well of a 96-well round bottom plate) were incubated with diluted primary antibody in predetermined optimal dilutions (WCI12 1:200; TCL-BE8 1:1000 and WCL15 1:100) for 30 min on ice and washed with flow

cytometer medium (incomplete medium supplemented with 1% bovine serum albumin (BSA) and 0.01% Na-azide). Subsequently, cells were incubated with a 1:50 dilution of a secondary antibody (rabbit-anti-mouse R-Phycoerythrin (RPE) and goat-anti-mouse fluorescein isothiocyanate (FITC) Dakopats, Glostrup, Denmark) for 30 min on ice, washed, and resuspended in 200 µl flow cytometer medium containing PI (0.1 µg/ml) to detect and gate out PI^+ cells. Per sample 10^4 events were measured by flow cytometer. A control sample incubated with the secondary antibody only, was included in each experiment and consistently found to be negative.

Light microscopy

For light microscopy, cell suspensions of freshly isolated HKL and head kidney-derived macrophages were pelleted (10 min 450 x *g*). Cell pellets were fixed in 1% (w/v) $K_2Cr_2O_7$, 2% (v/v) gluteraldehyde and 1% (w/v) OsO_4 in 0.1 M sodium cacodylate buffer, pH 7.2, for 1 hour at 0ºC, and subsequently washed in double-distilled water, dehydrated in alcohol and propylene oxide and embedded in Epon 812 (Electron Microscopy Science, Fort Washington, USA). Semi-thin sections were cut on a Reichert Ultracut E (Leica, Rijswijk, The Netherlands), stained with 1% (w/v) toluidine blue O, 1% (w/v) borax on a hot plate for 1 min, rinsed with tap water and embedded in depex (Serva, Heidelberg, Germany).

Phagocytosis, radical production, arginase activity and nitrite production

Freshly isolated HKL or head kidney-derived macrophages ($5x10^5$) were seeded in 100 µl rich-NMGFL-15 medium [incomplete-NMGFL-15 medium supplemented with 2.5% heat-inactivated pooled carp serum and 5% bovine calf serum (Invitrogen, Breda, The Netherlands)] in wells of a 96-well flat-bottom culture plate.

For measurement of phagocytosis, cells were stimulated with LPS (50 µg/ml; *Escherichia coli*, Sigma L2880), or left untreated to measure basal phagocytosis, and incubated for 18 hours at 27ºC. Stimulated cells were resuspended by pipetting, transferred to flow cytometry tubes and incubated with FITC-stained bacteria ($5x10^6$) for an additional hour at 27°C. Bacteria (*Staphylococcus aureus*) (Pansorbin, Calbiochem, EMD Biosciences, Darmstadt Germany) were stained by overnight incubation with FITC (5 µg/ml) at room temperature and continuous rotation. Bacteria were washed, at least three times, with incomplete medium to remove all unbound FITC. Phagocytosis was stopped by placing the tubes on ice and adding 1-2 ml ice-cold PBS. PI (0.1 µg/ml) was added to each sample to detect and gate out PI^+ cells, fluorescence of non-phagocytosed bacteria was quenched by adding trypan blue (130 µg/ml). A total number of 10^4 events in the cell gate were measured by flow cytometer, the cell gate excluded the free bacteria from the measurements.

For measurement of radical production cells were stimulated with phorbol 12-myristate 13-acetate (PMA) (0.1 µg/ml) or left untreated as controls. At the same time dihydrorhodamine 123 (DHR) (10 µg/ml Sigma D1054 -28.8 µM) was added to all samples, and samples were incubated for 1 hour at 27ºC. Cells were resuspended by pipetting, transferred to flow cytometry tubes and PI (0.1 µg/ml) was added to each sample to detect and gate out PI^+ cells, 10^4 events were measured by flow cytometer.

For measurement of arginase activity cells were stimulated with the cAMP analogue dibutyryl cAMP (0.5 mg/ml, dibutyryl cyclic adenosine mono phosphate, Sigma D0672), or left untreated, and incubated for 18 hours at 27ºC. Arginase activity was measured essentially as described by Corraliza et al. (27): cells were lysed in 50 µl 0.1% Triton X-100 containing 5 µg pepstatin, 5 µg aprotinin and 5 µg antipain at room temperature for 30 min. Fifty µl of 10 mM $MnCL_2$, 50 mM Tris-HCL, pH 7.5 was added and the mixture was incubated for 10 min at 55ºC. To 50 µl of this activated lysate 50 µl of 0.5 M L-arginine, pH 9.7 was added and incubated for 1 hour at 37ºC. Reaction was stopped by adding 400 µl acid mixture containing H_2SO_4, H_3PO_4 and H_2O (1:3:7), then to each reaction 25 µl 9% α-isonitrosopropiophenone (in 100% ethanol) was added and incubated for 45 min at 100ºC. After 10 min cooling in the dark the absorbance was read at 540 nm and arginase activity (mU per million cells, mU= nmol urea formed per min) was calculated by comparison with a urea standard curve.

For measurement of nitrite production, cells were stimulated with LPS (50 µg/ml), or left untreated, and incubated for 18 hours at 27ºC. Nitrite production was measured essentially as described by Green et al. (28): to 75 µl cell culture supernatant 100 µl 1% (w/v) sulphanilamide in 2.5% (v/v) phosphoric acid and 100 µl of 0.1% (w/v) N-naphthyl-ethylenediamine in 2.5% (v/v) phosphoric acid were added in a 96-well flat-bottom plate. The absorbance was read at 540 nm (with 690 nm as a reference) and nitrite concentrations (µM) were calculated by comparison with a sodium nitrite standard curve.

Gene expression profiling of LPS and cAMP stimulated head kidney-derived macrophages

To analyze the functional heterogeneity of head kidney-derived macrophages, gene expression levels of an array of genes (TNFα, IL-11, IL-1β, IL-1βR, IL-10, IL-12P35, IL-12P40.1, IL-12P40.2, IL-12P40.3, iNOS, arginase 1, arginase 2, CXCa, CXCb, CXCR1, CXCR2, MHC-II, NILT1 and NILT2) were measured after LPS or cAMP stimulation. Head kidney-derived macrophages were stimulated with LPS (50 µg/ml) or cAMP (0.5 mg/ml) or left untreated as control, and incubated for 6 hours at 27ºC. To study NILT gene expression in more detail, samples were taken after 0, 3, 6, 9 and 18 hours of incubation at 27ºC. From all samples RNA was isolated, cDNA synthesized and gene expression levels were determined by means of real-time quantitative PCR (see later sections).

Gene expression during Trypanosoma carassii and Trypanoplasma borreli infections

Trypanosoma carassii was cloned and characterized by *Overath et al.* (29). *Trypanoplasma borreli* was cloned and characterized by Steinhagen *et al.* (30). Parasites were maintained by syringe passage through carp. Thirty-two carp were i.p. injected with 10,000 *Trypanosoma carassii* per fish and four were left untreated as control. In a separate experiment twenty carp were i.p. injected with 10,000 *Trypanoplasma borreli* per fish and five were left untreated as control. At every time point four fish for the *T. carassii* and five fish for the *T. borreli* experiment were sacrificed and their head kidneys removed. Head kidneys were

snap frozen in liquid nitrogen and stored at -80ºC. From all head kidneys RNA was isolated, cDNA synthesized and gene expression levels were determined by means of real-time quantitative PCR (see later sections).

RNA isolation, DNase treatment and first strand cDNA synthesis

RNA was isolated from a 27 mm^3 head kidney (organ) or $2x10^6$-$5x10^6$ cells per treatment using the RNeasy Mini Kit (Qiagen, Leusden, The Netherlands) including the accompanying DNase I treatment on the columns, according to the manufacturer's protocol. End elution was performed with 25 µl diethyl pyrocarbonat (DEPC)-treated water. RNA concentrations were measured by spectrophotometry (Genequant, Pharmacia Biotech, Uppsala, Sweden) and 1 µl was analyzed on a 1% agarose gel to check the integrity. Routinely, 10 µg of RNA approximately was isolated from 27 mm^3 head kidney (organ) and 15 µg of RNA from 2-$5x10^6$ cells. RNA was stored at -80ºC until further use. For each cDNA synthesis a negative sample (non-RT), to which no reverse transcriptase was added and a positive sample containing the reverse transcriptase were included. After DNAse treatment, 1 µg of total RNA was combined with, random primers (300 ng Invitrogen, Breda, The Netherlands), 1 µl dNTP mix (10 mM), 4 µl First Strand buffer (5x), 2 µl DTT (0.1 M) and 1 µl RNase inhibitor (40 U/µl Invitrogen, Breda, The Netherlands) and the mix was incubated at room temperature for 10 min. To each positive sample, 1 µl Superscript RNase H Reverse Transcriptase II (200 U/µl Invitrogen, Breda, The Netherlands) was added. To each negative sample, 1 µl DEPC-treated water was added. All samples were incubated at 37ºC for 50 min. Reactions were stopped by adding MilliQ water up to 100 µl and cDNA stored at -20ºC until use.

Real-time quantitative PCR

Specific real-time quantitative PCR primers (Table I) were designed with the Primer Express software (Applied Biosystems, Foster City, CA, USA). To 5 µl of 10 times-diluted cDNA, 7 µl Sybr Green Master Mix (Stratagene, La Jolla, CA, USA), forward and reverse primer (300 nM each) and MilliQ water up to 14 µl was added. Quantitative PCR was performed in a 72-well Rotor-Gene™ centrifugal real-time thermal cycler (Rotor-Gene 2000 Corbett Research, Sydney, NSW, Australia). Following cycling conditions were used: one holding step of 10 min at 95ºC; followed by 40 cycles of 20 sec at 94ºC, 30 sec at 60ºC and 30 sec at 72ºC; an incubation for 1 min at 60ºC was followed by a melting curve from 60ºC to 99ºC in steps of 1ºC with 5 sec waiting. At the end of each cycle and during the waiting steps in the melting curve, fluorescence intensities were measured. Raw data were analyzed using the comparative quantitation of the Rotor-Gene Analysis Software V5.0. Data were further analyzed using the Pfaffl method (31), average efficiencies per run per gene were used. Gene expression of 40S in each sample was highly constant and used to normalize the data. Specificity of the amplification was ensured by checking the melting temperature and profile of each melting curve. The product of each template was checked at least once by sequencing.

Table I. Primer sequences corresponding accession numbers, real-time quantitative PCR efficiencies, and melting temperatures

Gene	Acc. No.	Sense (5'-3')	Antisense (5'-3')	Melt temp	Eff.
TNFα	AJ311800-01	GCTGTCTGCTTCACGCTCAA	CCTTGGAAGTGACATTTGCTTTT	78.0	1.72
IL-1β	AJ245635	AAGGAGGCCAGTGGCTCTGT	CCTGAAGAAGAGGAGGCTGTCA	78.8	1.74
IL-1R	AJ843873	ACGCCACCAAGAGCCTTTTA	GCAGCCCATATTTGGTCAGA	76.7	1.75
IL-10	AB110780	CGCCAGCATAAAGAACTCGT	TGCCAAATACTGCTCGATGT	74.5	1.86
IL-11	AJ632159	CAGCAGCACAGCTCAGTACCA	AGCCTCTGCTCGGGTCATCT	78.8	1.74
IL-12P35	AJ580354	TGCTTCTCTGTCTCTGTGATGGA	CACAGCTGCAGTCGTTCTTGA	77.3	1.86
IL-12P40.1	AJ621425	GAGCGCATCAACCTGACCAT	AGGATCGTGGATATGTGACCTCTAC	77.0	1.79
IL12P40.2	AJ628699	TCTTGCACCGCAAGAAACTATG	TGCAGTTGATGAGACTAGAGTTTCG	76.7	1.41
IL12P40.3	AJ628700	TGGTTGATAAGGTTCACCCTTCTC	TATCTGTTCTACAGGTCAGGGTAACG	77.3	1.85
iNOS	AJ242906	AACAGGTCTGAAAGGGAATCCA	CATTATCTCTCATGTCCAGAGTCTCTTCT	77.7	1.69
Arginase 1	AJ871264	TGAGGAGCTTCAGCGGATTAC	CCTATTATTCCCACGCAGTGATG	77.3	1.76
Arginase 2	&	GGAGACCTGGCCTTCAAGCATCT	CTGATTGGCACGTCCAACT	80.3	1.73
CXCa	AJ421443	CTGGGATTCCTGACCATTGGT	GTTGGCTCTCTGTTTCAATGCA	78.8	1.78
CXCb	AB082985	GGGCAGGTGTTTTTGTGTTGA	AAGAGCGACTTGCGGGTATG	77.3	1.76
CXCR1	AB010468	GCAAATTGGTTAGCCTGGTGA	AGGCGACTCCACTGCACAA	80.8	1.75
CXCR2	AB010713	TATGTGCAAACTGATTTCAGGCTTAC	GCACACACTATACCAACCAGATGG	80.5	1.82
MHC-II DAB1-2	Z47731-32	ACAGCTCCCGTGATTTCAGT	CTCTGCGTTATATACTCCAAGTGC	77.0	1.85
MHC-II DAB3-4	Z47733/X95435	GCGTTTCAGGCGGACTCTT	ACACCATATCACTGTAATCACT	77.3	1.60
NILT1	AJ811996	CATACTCTGATTTCTGGACACAGA	CTGTTTTACCAGCAACAAAATCTC	77.8	1.69
NILT2	AJ811997	GGTGCTCCAGTTAAAGTCACAGGA	CTGTTTAACCAGCAACACAATCTC	79.5	1.74
40S	AB012087	CCGTGGGTGACATCGTTACA	TCAGGACATTGAACCTCACTGTCT	78.5	1.77
β-actin	M24113	GCTATGTGGCTCTTGACTTCGA	CCGTCAGGCAGCTCATAGCT	81.8	1.73

& there are three arginase 2 isoforms known AJ618955/AJ871265-66 this primer set amplifies all three.

Statistics

Phagocytosis, radical production, arginase activity, nitrite production and gene expression differences between the differential stimulated head kidney-derived macrophages and during the infections were tested for significance by Student's *t* test. $p<0.05$ was accepted as significant.

NILT1 and NILT2 gene expression in the head kidney-derived macrophages over time, were analyzed by a repeated measurement model (PROC GLM, SAS, version 8.02, SAS Inst., Inc., Cary NC)(32), with time as the repeating factor. The treatments were tested for significance against the interaction of treatment and individual. $p<0.05$ was accepted as significant.

RESULTS

Macrophage cell culture: characterization by cell number, surface marker staining and morphology

Carp head kidney leukocytes (HKL) were separated on a 51% Percoll layer and cultured *in vitro* for several days. A pilot experiment where cells were cultured up to 8 days and analyzed by flow cytometer indicated an expanding cell population until day 7. To characterize these *in vitro* cell cultures we followed their development by flow cytometry, determining cell number, size and internal complexity (forward scatter (FSC-H) and sideward scatter (SSC-H), respectively) and expression of cell surface markers up to day 6 in culture.

During the first 2 days of culture, total cell numbers decreased drastically, while after 4 days of culture cell numbers increased again, indicating proliferation (Fig. 1A). At the start of the culture (day 0), three cell populations could be identified based on FSC-H/SSC-H dot plots (Fig. 1B). First, a population of lymphocytes and precursor cells was characterized by a low FSC-H/SSC-H (lower left hand corner). Most of the WCI12$^+$ cells (B lymphocytes) could be found in this population. Over time, during the first two days of culture especially, this lymphocyte population decreased dramatically (Figs 1B and 1C). Second, a population of monocytes was characterized by a medium FSC-H/SSC-H (spreading out to the middle). A low percentage of WCL15$^+$ cells (macrophages, monocytes, basophilic granulocytes) could be found primarily in this population. Over time, the relative percentage of WCL15$^+$ cells increased steadily (Fig. 1C). Third, a population of neutrophilic granulocytes was characterized by a high FSC-H/SSC-H (upper right hand corner). Almost all of these cells were TCL-BE8$^+$ (neutrophilic granulocytes, monocytes). During the first two days of culture the relative percentage of TCL-BE8$^+$ cells remained constant (Fig. 1C). However, since the total cell number decreased rapidly (Fig. 1A), the absolute number of TCL-BE8$^+$ cells also decreased. From day 4 onwards, the percentage of TCL-BE8$^+$ cells increased again.

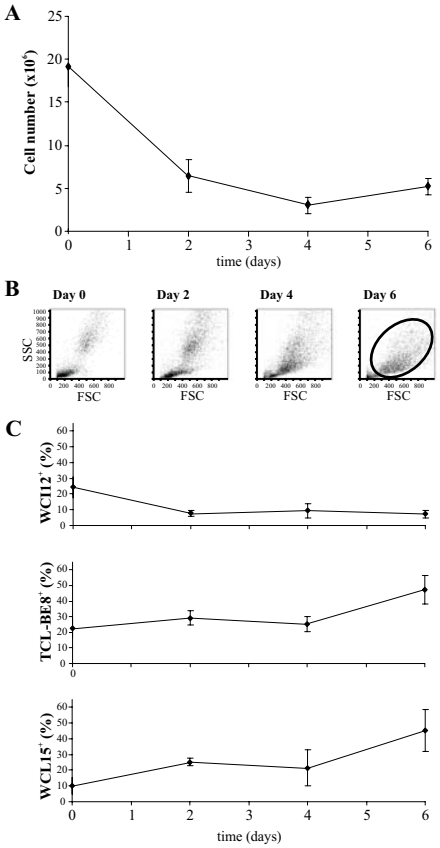

Figure 1. Flow cytometric analyses of carp head kidney leukocyte cultures. Head kidney leukocytes were cultured in complete NMGFL-15 medium. At day 0, 2, 4 and 6 cells were collected to determine: (A) cell numbers per culture, (B) cell size and internal complexity and (C) surface marker staining. To determine cell numbers 100 µl of a standard bead solution was added and cells with beads were analyzed by flow cytometry. Averages and SD of n=3 fish are given. To determine cell morphology, dot plot profiles for forward scatter (FSC-H, reflecting size) and sideward scatter (SSC-H, reflecting internal complexity) were measured. Data from a representative experiment out of n=8 fish are shown. For cell surface marker staining the monoclonals WCI12 (1:200), TCL-BE8 (1:1000), and WCL15 (1:100) were used. Averages and SD of n=4 fish are given. For all flow cytrometric measurements PI (0.1 µg/ml) was included to detect and gate out PI$^+$ cells.

After four days, cell cultures consisted of two populations (Fig. 1B). One major population of head kidney-derived macrophages with a high FSC-H/SSC-H that were TCL-BE8$^+$, WCL15$^+$, or TCL-BE8$^+$WCL15$^+$, as confirmed by fluorescence microscopy (data not shown). The second population was smaller in number and consisted of precursor cells with a low FSC-H/SSC-H that were negative for all three cell surface markers used. During the last two days of the culture especially, the head kidney-derived macrophages further increased in number, both relative and absolute. Changes in cell number were also reflected by the changes in the percentage of PI$^+$ cells. The percentage of PI$^+$ cells increased from 5% at day 0 to 10% at day 2, but decreased again to 7% at day 6.

Light microscopy (Fig. 2) supported the decrease in lymphocytes and granulocytes and the increase in myeloid cells as detected by the antibody labeling. Within this myeloid cell population there is still a degree of heterogeneity visible.

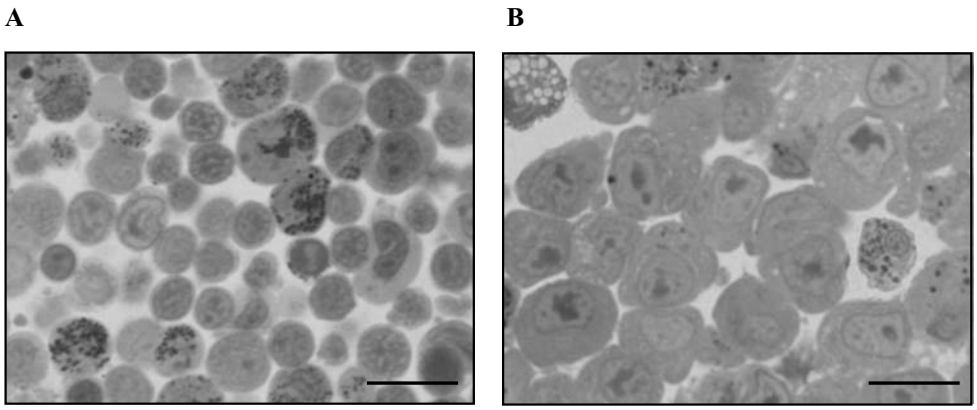

Figure 2. Light microscopic photographs (bar is 10 μm) of freshly isolated head kidney leukocytes (A) and head kidney-derived macrophages (B). Cells were pelleted and fixed, semi-thin sections were cut and stained with toluidine blue and borax.

Phagocytic ability, (oxygen) radical production, arginase activity and nitrite production are high in head kidney-derived macrophages

Carp HKL were cultured *in vitro* for 6 days to obtain head kidney-derived macrophages, which were assessed for cell function by measuring phagocytosis and (oxygen) radical production by flow cytometry and arginase activity and nitrite production by a calorimetric method. The phagocytic ability, the number of cells capable of phagocytosis, was measured both in freshly isolated HKL (day 0) and in head kidney-derived macrophages (day 6). In a pilot experiment, a time curve showed a plateau phase of phagocytosis after 1 hour incubation at 27ºC, which was chosen as the time of incubation in the following experiments. Head kidney-derived macrophages showed significantly higher basal phagocytic ability (19%) of FITC-labeled bacteria, as compared to freshly isolated HKL (8%). Pre-stimulation with LPS for 18 hours further increased the phagocytic ability both in head kidney-derived macrophages (23%) and in freshly isolated HKL (13%) (Fig. 3A).

Radical production was measured by flow cytometry following incubating with DHR. Head kidney-derived macrophages showed a very high (77%) basal activity, as compared to freshly isolated HKL (22%) (Fig. 3B). While radical production in freshly isolated HKL could be significantly increased by PMA stimulation (51%), radical production by head kidney-derived macrophages could not be further increased (79%).

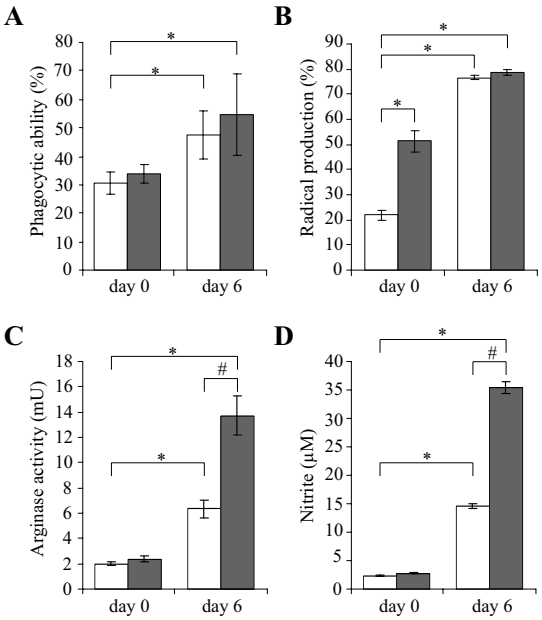

Figure 3. Phagocytosis (A), radical production ($^{\bullet}OH$, $CO_3^{\bullet-}$, NO_3^-, H_2O_2 and $NO_2^{\bullet}$) (B), arginase activity (C) and nitrite production (D) by freshly isolated head kidney leukocytes (day 0) and head kidney-derived macrophages (day 6). Cells ($5x10^5$ per 100 µl) were stimulated (closed bars) or left untreated to measure basal activity (open bars). Cells were stimulated with LPS (50 µg/ml) for phagocytosis (A) and nitrite production (D), with PMA (0.1 µg/ml) for radical production (B) or with cAMP (0.5 mg/ml) for arginase activity (C). Averages and SD of n=4 fish are given for phagocytosis and radical production. Averages and SD or triplicate measurements of a representative experiment out of n=7 are given for arginase activity and nitrite production. *, $p<0.05$, by Student's *t* test, compared to day 0 control. #, $p<0.05$ Student's *t* test, compared to corresponding time point control.

Arginase activity was measured after 18 hours of incubation with or without cAMP stimulation. Head kidney-derived macrophages showed a significantly higher arginase activity, as compared to freshly isolated HKL (Fig. 3C). This was true both for basal activity (2 versus 6 mU) as well as for cAMP-induced activity (2 versus 14 mU). cAMP stimulation increased the arginase activity of head kidney-derived macrophages significantly.

Nitrite production was measured after 18 hours of incubation with or without LPS stimulation. Head kidney-derived macrophages showed a significantly higher ability to produce nitric oxide, measured as nitrite in a Griess reaction, as compared to freshly isolated HKL (Fig. 3D). This was true for both basal activity (2 versus 15 µM) and for LPS-induced activity (3 versus 36 µM). LPS stimulation increased the NO production of head

kidney-derived macrophages significantly. Basal nitric oxide levels were high in head kidney-derived macrophages, considering a stimulation with LPS of 18 hours only, already showed a considerable nitrite production of 15 μM.

Gene expression profiles of LPS- or cAMP-stimulated head kidney-derived macrophages are different

RNA was isolated from head kidney-derived macrophages (6 day culture) stimulated for 6 hours with LPS or cAMP or from unstimulated cells, to assess differences in gene expression as a measure of macrophage polarization. Gene expression levels of an array of immune-relevant genes currently described for carp were studied by real-time quantitative PCR, using 40S and β-actin gene expression as house keeping gene references. Results with 40S and β-actin were comparable; only the results relative to 40S are shown (Table II).

Table II. Gene expression of head-kidney-derived macrophages after 6h stimulation with LPS or cAMP relative to unstimulated cells

Gene	Take off value[&]	LPS	cAMP
TNFα[#]	21.2	1.14*	0.74
IL-1β	24.9	6.62*	5.24*
IL-1R	22.8	1.33	1.10
IL-10[#]	21.4	1.01	0.60*
IL-11	27.5	1.72	2.33
IL-12P35	27.8	2.04*	1.93
IL-12P40.1	29.2	1.27	0.75
IL-12P40.2	27.3	1.27	1.19
IL-12P40.3[#]	22.7	1.53	3.04*
iNOS[#]	25.5	8.08*	0.74
Arginase 1	30.2	1.31	0.79
Arginase 2[#]	21.4	1.33	3.08*
CXCa[#]	24.0	1.56*	0.91
CXCb	29.5	1.17	1.00
CXCR1[#]	27.3	0.73	7.18*
CXCR2[#]	25.8	1.26	3.46*
MHC-II DAB1-2	18.1	0.94	0.91*
MHC-II-DAB3-4	28.2	1.50	1.32
NILT1	31.8	1.07	2.05*
NILT2[#]	22.3	1.22*	0.81*

& Take off value is the point 80% before the peak of the second derivative of the raw data (Rotor-Gene Analysis Software V5.0).

Significant difference between the LPS and cAMP stimulated cells.

* Significant difference compared to the unstimulated cells.

Gene expression levels of cells stimulated with LPS or cAMP were compared to unstimulated cells, as shown in Table II. In addition, gene expression levels of LPS-stimulated head kidney-derived macrophages were compared with gene expression levels in cAMP-stimulated head kidney-derived macrophages. From the differentially expressed genes, TNFα, IL-10, iNOS, CXCa, and NILT2 were significantly higher expressed in LPS-stimulated head kidney-derived macrophages, while IL12P40.3, arginase 2, CXCR1, and CXCR2 were significantly higher expressed in cAMP-stimulated head kidney-derived macrophages.

NILT1 gene expression is upregulated in cAMP-stimulated head kidney-derived macrophages and in Trypanosoma carassii-infected carp but not in Trypanoplasma borreli-infected carp

From the genes differentially expressed in LPS- or cAMP-stimulated head kidney-derived macrophages we selected the NILT genes for a more detailed study because of the unknown function of these novel receptors in fish. To further characterize the gene expression of NILT1 and NILT2, head kidney-derived macrophages were stimulated with LPS, with cAMP, or left untreated as control and gene expression relative to 40S was followed over time. NILT1 gene expression was significantly upregulated after stimulation with cAMP. In contrast, LPS did not influence NILT1 expression (Fig. 4A).

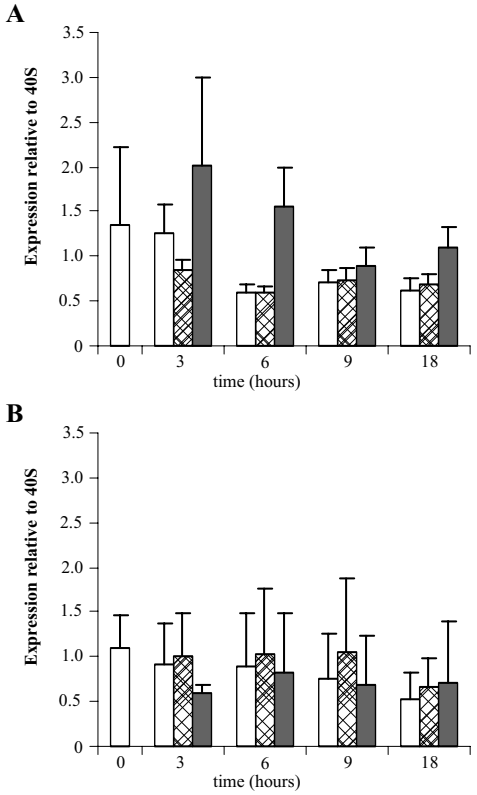

Figure 4. Gene expression of NILT1 (A) and NILT2 (B) in head kidney-derived macrophages. Head kidney-derived macrophages, $5x10^5$ per well, were stimulated with LPS (50 µg/ml) (hatched bars), cAMP (0.5 mg/ml) (solid bars) or left untreated as control (open bars). Gene expression was measured by means of real-time quantitative PCR and is shown relative to the gene expression of 40S. Averages and SD of n=3 fish are given. Statistical differences were calculated by a repeated measurement model with time as a repeating factor. NILT1 gene expression in the cAMP stimulated head kidney-derived macrophages is significantly different from both the LPS stimulated and unstimulated head kidney-derived macrophages. NILT2 gene expression in the cAMP stimulated head kidney-derived macrophages is significantly different from the LPS stimulated head kidney-derived macrophages.

NILT2 gene expression was consistently upregulated after LPS stimulation. cAMP stimulation showed a consistent downregulation of NILT2 (Fig. 4B).

In addition, we measured the *in vivo* gene expression of both NILT1 and NILT2 in head kidney (whole organ) of carp infected with either *Trypanosoma carasssii* or *Trypanoplasma borreli*. Both NILT1 and NILT2 gene expression were downregulated during the early phase of infection with *T. carassii* but upregulated during later time points in the *T. carassii* infection (Figs 5A and 5C). Observed effects were stronger for NILT1 than for NILT2. The *T. borreli* infection was more severe and lasted shorter than the *T. carassii* infection. During *T. borreli* infection, NILT gene expression was different. NILT1 gene expression was significantly upregulated only at week 4 (Fig. 5B), while NILT2 gene expression was not significantly regulated (Fig. 5D).

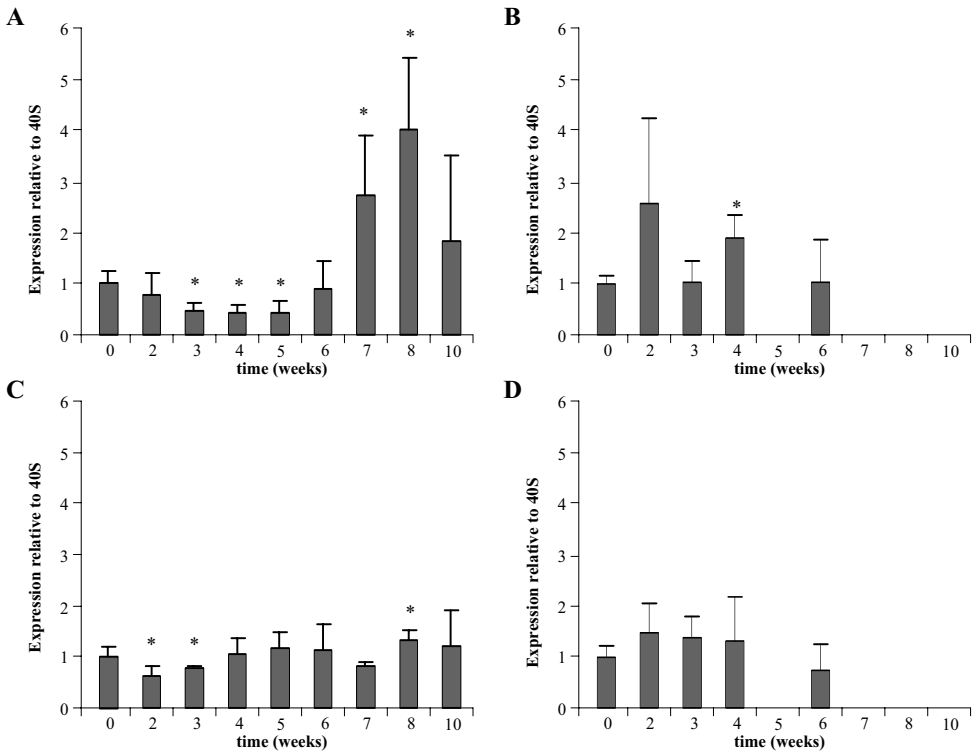

Figure 5. Gene expression of NILT1 (A and B) and NILT2 (C and D) in head kidney (organ) of *Trypanosoma carassii*-infected carp (A and C), or *Trypanoplasma borreli*-infected carp (B and D). A number of n=32 carp were i.p. infected with 10,000 *T. carassii* per fish and a number of n=20 carp were i.p. infected with 10,000 *T. borreli* per fish. At week 0, the week of infection, n=4-5 carp were sacrificed as uninfected control fish. At every sample point n=4 fish from the *T. carassii* and n=5 fish from the *T. borreli* infection were sacrificed, head kidneys were removed, snap frozen in liquid nitrogen and stored at -80ºC. Gene expression was measured by means of real-time quantitative PCR and is shown relative to the gene expression of 40S. Averages and SD of n=4-5 fish are given. *, $p<0.05$, by Student's *t* test, compared to uninfected control.

Discussion

Our experiments show that carp head kidney-derived macrophages are heterogeneous and upon *in vitro* stimulation can give rise to functionally different polarization states. According to FSC-H/SSC-H profiles the cell cultures consisted of two populations. The majority of the cells (>80%) with a high FSC-H/SSC-H profile are mature macrophages, as discussed in detail later. The remainder of the cells with a low FSC-H/SSC-H profile most likely are precursor cells of the myeloid lineage, as was shown for goldfish (33). The functional heterogeneity gives rise to macrophage populations that can be activated innate or alternatively. Gene expression profiling suggested that NILT1, CXCR1 and CXCR2 might be used as surface markers for alternatively activated macrophages. The use of NILT1 as putative cell surface maker for aaMF in fish was confirmed in an *in vivo* experiment.

Until present, carp macrophages have been studied using head kidney-derived leukocytes isolated by Percoll density centrifugation, followed by an adherence step in RPMI medium with adjusted molarity. This method leads to slightly-enriched fractions of macrophages (23). To study macrophage polarization and functional heterogeneity of a primitive vertebrate in more detail we here describe a method to culture carp macrophages, based on a procedure previously developed for goldfish and trout (17-19). Head kidney leukocytes (HKL) were isolated on a 51% Percoll layer, seeded in NMGFL-15 medium (17) and cultured for six days. NMGFL-15 medium enhances myeloid but not lymphoid cell growth of goldfish and trout (17, 18). Indeed, also for carp the NMGFL-15 medium leads to an early reduction in the number of lymphocytes ($WCI12^+$ (23), low FSC-H/SSC-H (24)) and supported a subsequent increase in the number of macrophages ($WCL15^+TCL\text{-}BE8^+$ (25) (26), high FSC-H/SSC-H; (19)). Light microscopy supported head kidney-derived cell cultures to be primarily macrophages with phenotypic heterogeneity.

Head kidney-derived macrophages present at day 6 of culture were functional macrophages as could be seen from their apparent capacity to phagocytose fluorescent bacteria and increased radical production. To measure the ROS production cells were incubated with DHR. Free radicals and some other molecules ($^{\bullet}OH$, $CO_3^{\bullet-}$, NO_3^-, H_2O_2 and $NO_2^{\bullet}$) can oxidize the nonfluorescent DHR into the highly green fluorescent rhodamine 123 which localizes in the mitochondria (34). DHR is not oxidized by $O_2^{\bullet-}$ (35, 36). The radical $O_2^{\bullet-}$ can, however, react with other molecules to form radicals that can be measured by DHR (35). The low radical production by freshly isolated HKL could be ascribed, based on FSC-H/SSC-H, primarily to neutrophilic granulocytes and to a lesser extend to monocytes. In contrast, we observed a very high spontaneous radical production in carp head kidney-derived macrophages. Also, when radical production was measured using reduction of nitro blue tetrazolium (NBT), similar results were observed. In fact, for freshly isolated HKL, 90 minutes incubation was needed to obtain a substantial amount of NBT reduction, while head kidney-derived macrophages accounted for two times higher NBT reduction within 30 minutes only (unpublished data). Most likely, this high basal activity is caused by dead cells and / or cytokines also present in the cell cultures. In conclusion, cultured carp

head kidney-derived macrophages provide us with an excellent model to investigate the polarization and functional heterogeneity of fish macrophages.

There is a growing interest in the role and functioning of alternatively activated macrophages. Recently, we described the possible conservation of alternative macrophage activation down to teleost fish (16). In the present study we used cAMP to stimulate arginase activity, as a measure of alternative activation. Both NO production and arginase activity were higher in head kidney-derived macrophages than in freshly isolated HKL. Furthermore, we observed a clear increase of NO production or arginase activity after stimulation with LPS, or cAMP, respectively. This increase in activity suggested that our culture system leads to enrichment for macrophages which are still able to polarize into either innate activated macrophages or alternatively activated macrophages.

For fish macrophages, it may be difficult to distinguish between innate and classically activated macrophages, since LPS sensitivity and subsequent intracellular signaling are recognized to be different from the mammalian situation (37). Fish cells not only require high amounts of LPS for stimulation they also seem to lack several proteins involved in the LPS signaling pathway (37). In fish, LPS is believed to signal via beta-2 integrins and not through toll-like receptor 4 (37). True classical activation of macrophages would also require stimulation with IFNγ. Previous experiments in our laboratory, using macrophage-activating factor (supernatants from mitogen-stimulated cells, believed to contain IFNγ or IFNγ-like proteins) in addition to LPS, did not result in an additional stimulation of nitrite production, neither in cultured nor in freshly isolated macrophages. We therefore believe the present NO response is due to innate rather than classical activation. Recently, the first fish IFNγ sequences were identified and characterized (38, 39) and, in time, production of recombinant fish IFNγ might allow for a further distinction between innate and classical activation of fish macrophages.

To further assess the polarization of head kidney-derived macrophages we measured the gene expression of an array of immune-relevant genes after differential stimulation. Gene expression profiles of caMF and aaMF in both human and mouse are relative well studied and extensively reviewed by Mantovani *et al.* (3, 40). From the differentially expressed genes in carp, TNFα, IL-10, iNOS, CXCa, and NILT2 were significantly higher expressed in LPS-stimulated head kidney-derived macrophages. IL12P40.3, arginase 2, CXCR1 and CXCR2 were significantly higher expressed in cAMP-stimulated head kidney-derived macrophages. The upregulation of TNFα and iNOS after LPS stimulation (innate activation) is similar to the mammalian situation. It is more difficult to compare the CXC gene expression with the mammalian situation, since phylogenetic analyses could not assign carp CXC chemokines according to the human CXC nomenclature (41). CXCL8-11 and CXCL-16 are typically upregulated in mammalian caMF, similarly, CXCa was upregulated in the LPS-stimulated head kidney-derived macrophages. The, in comparison, higher gene expression of IL-10 in LPS-stimulated macrophages could be primarily ascribed to a downregulation of IL-10 in cAMP-stimulated macrophages. This is different from the mammalian situation. The expression of IL-10 in fish certainly requires further study (42). The expression of

IL-12 is difficult to compare between different species since expression and translation of IL-12P35 and IL-12P40 are tightly, but differently, regulated even between human and mouse, as reviewed by Trinchieri (43). Expression can be regulated by both classical activators such as IFNγ, but also by alternative activators like IL-4 and IL-13. Here, we describe an upregulation of IL-12P40.3 gene expression after stimulation with cAMP, an alternative activator of fish macrophages. The upregulation of arginase, CXCR1 and CXCR2 after cAMP stimulation (alternative activation) is comparable to the mammalian situation where CXCR1 and CXCR2 gene expression are upregulated in alternatively activated macrophages (3). Increased arginase expression in alternatively activated macrophages confirms our previous findings (16), the increased CXCR1 and CXCR2 gene expression is, however, a new finding. We suggest that CXCR1 and CXCR2 might be useful new surface markers for alternative macrophage activation in fish.

This is the first report on a differential expression of NILT genes. The recently described NILT genes show significant similarity to the human triggering receptors expressed on myeloid cells (TREM), to CMRF35 and to the natural cytotoxicity receptor NKp44. The location of zebrafish NILT homologues on chromosome 1 at 7 Mb downstream of the MHC class I cluster, suggests that the carp NILT genes are related to either the NKp44 or the TREM genes (20). Expression of NKp44 is restricted to NK cells (44), while TREMs are expressed on different cells of the myeloid lineage (45-48). Preliminary studies of NILT expression on carp peripheral blood lymphocytes were inconclusive as to ascribe a function to these genes (20). Here, we show both NILT1 and NILT2 expression in head kidney-derived macrophages, cells of the myeloid lineage, suggesting a close relationship to TREM.

The TREM gene family comprises at least six members of which TREM-1 and -2 are best studied. TREM-1 expression is upregulated in myeloid cells after LPS stimulation (45), or during sepsis (49). TREM-2 expression is associated with regulating myeloid lineage development (50). TREM-2 is also believed to play a role in chronic inflammation and may stimulate production of constitutive rather than inflammatory chemokines and cytokines (45, 46). Typically, aaMF are associated with chronic inflammation (51). In parallel, TREM-2 was shown to be upregulated during chronic infections of mice with African trypanosomes (52), together with other markers for alternative activation (53, 54).

We have shown an upregulation of NILT1 in head kidney-derived macrophages stimulated with cAMP, an inducer of alternative activation in carp macrophages (16). Furthermore, NILT1 gene expression was upregulated during the later time points of infection with *Trypanosoma carassii*. Recent *in vivo* experiments suggest that, during infection with *T. carassii*, macrophages are predominantly alternatively activated during the late phase of infection (Joerink *et al*., submitted for publication). In addition, NILT1 gene expression was hardly upregulated during an infection with *Trypanoplasma borreli,* where macrophages are primarily innate activated (10, 11). Currently we are developing specific antibodies against the NILT and CXCR proteins to unequivocally assign these proteins as surface markers of fish macrophages. We suggest that the NILTs might be TREM

homologues and that NILT1, together with CXCR1 and CXCR2, could likely be used as novel surface markers for alternatively activated macrophages in fish.

ACKNOWLEDGEMENTS

The authors would like to thank the central fish facilities, "De Haar-Vissen" for providing and taking care of the carp. Prof. Dr. M. Belosevic and J.L. Stafford are gratefully acknowledged for their enthusiastic support on establishing carp macrophage cultures. Ir. B. Ducro is acknowledged for his help with the statistical analysis of the gene expression data. Prof. N. Okamoto (Department of Aquatic Biosciences, Tokyo University of Fisheries, Tokyo, Japan) is acknowledged for providing the mouse monoclonal antibody TCL-BE8. Maria Forlenza, Mark Huising, Geert Raes, Nico Taverne, Anja Taverne-Thiele and Beja de Vries are gratefully acknowledged for their practical assistance or fruitful discussions.

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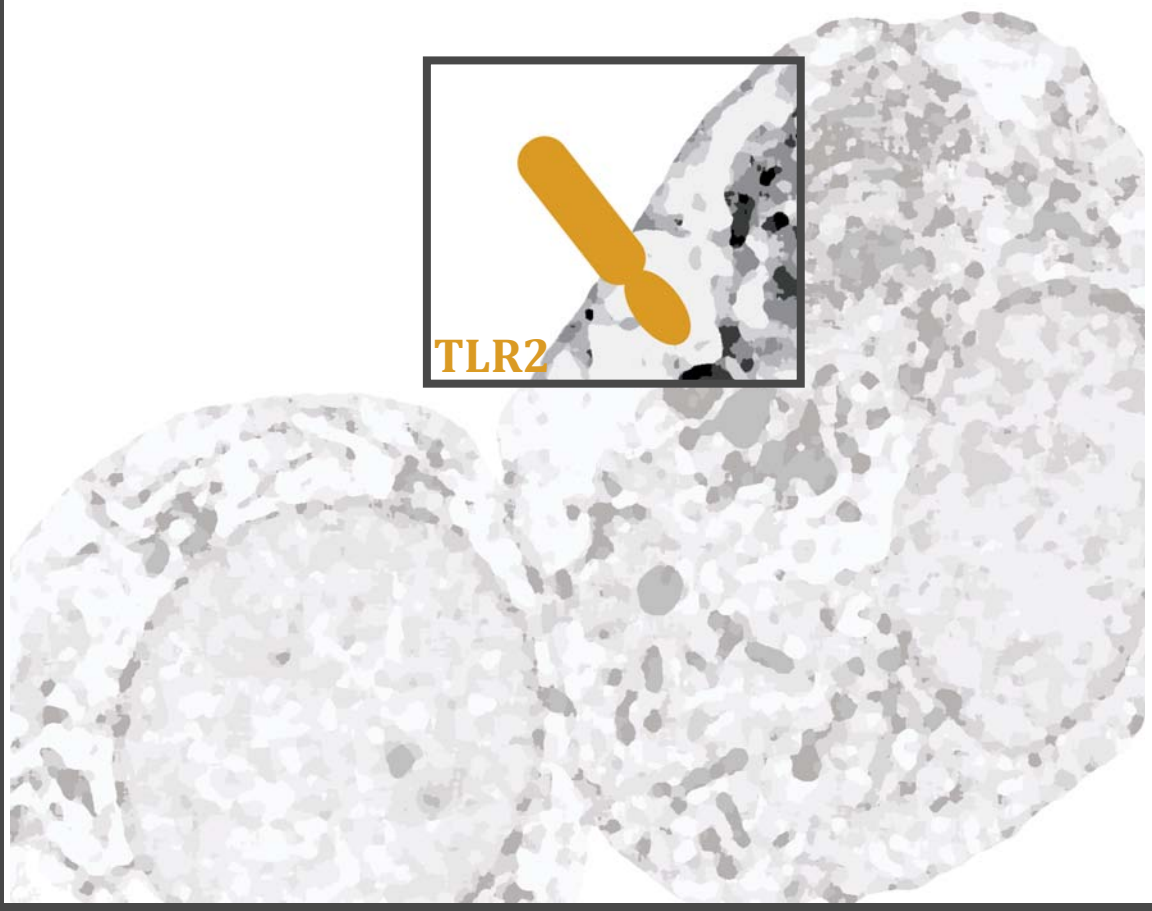
" You may say that I´m a dreamer
But I´m not the only one."

John Lennon



Chapter 4

Evolution of recognition of ligands from Gram-positive bacteria: similarities and differences in the TLR2-mediated response between mammalian vertebrates and teleost fish

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Journal of Immunology, 2010 (184), 2355-2368

ABSTRACT

We investigated the role of the TLR2 receptor in the recognition of ligands from Gram-positive bacteria in fish. Comparative sequence analysis showed a highly conserved TIR domain. Although the LRR domain was less conserved, the position of the critical PGN-binding residues in the LRR domain of carp TLR2 were conserved. Transfection of human HEK 293 cells with TLR2 corroborated the ability of carp TLR2 to bind the prototypical mammalian vertebrate TLR2 ligands LTA and PGN from *Staphylococcus aureus*. The synthethic triacylated lipopeptide Pam_3CSK_4 but not the diacylated lipopeptide MALP-2 also activated TLR2 transfected human cells. We identified clear differences between the mammalian vertebrates and carp TLR2-mediated response. The use of the same ligands on carp macrophages indicated that fish cells require high concentrations of ligands from Gram-positive bacteria (LTA, PGN) for activation and signal transduction, react less strongly (Pam_3CSK_4) or do not react at all (MALP-2). Overexpression of TLR2 in carp macrophages confirmed TLR2-reactivity of the response to LTA and PGN, low-responsiveness to Pam_3CSK_4 and non-responsiveness to MALP-2. A putative relation with the apparent absence of accessory proteins such as CD14 from the fish TLR2-containing receptor complex is discussed. Moreover, activation of carp macrophages by PGN resulted in increased TLR2 gene expression and enhanced TLR2 mRNA stability, MAPK-p38 phosphorylation and increased radical production. Finally, we could show that NAD(P)H oxidase-derived radicals and MAPK-p38 activation cooperatively determine the level of PGN-induced TLR2 gene expression. We propose that the H_2O_2-MAPK-p38-dependent axis is crucial for regulation of TLR2 gene expression in fish macrophages.

INTRODUCTION

The innate immune system of vertebrates uses pattern recognition receptors (PRRs) to sense invading pathogens. Toll-like receptors (TLRs) act as PRRs, able to recognize pathogen associated molecular patterns (PAMPs) that are conserved molecular structures and unique of a given microbial class (bacteria, viruses, fungi and protozoa), thereby allowing sufficient responses to limit or eradicate invading microbes. TLRs are type I transmembrane proteins with an ectodomain containing interspersed leucine-rich repeat (LRR) motifs involved in recognition of PAMPs. The cytoplasmic domain is characterized by a Toll/ IL-1 receptor (TIR) motif which is involved in signal transduction (1, 2). In mammalian vertebrates, TLR2 senses bacterial lipopeptides (3-5) and lipoteichoic acids (6, 7) as well as glycosylphosphatidylinositol anchors from parasites (8, 9). TLR2 recognizes microbial patterns as homodimer (10) and as heterodimers with TLR1 or TLR6 (11, 12). Expression of TLR2 in humans is highest in myeloid cells (monocytes, macrophages, dendritic cells and granulocytes) whereas in mice TLR2 expression was also found in T cells (13-15). Investigations of TLR-mediated immune recognition in non-mammalian vertebrates such

as teleost (modern bony) fish can provide new insights into the evolution of immunity (16). Research on teleost fish TLR2 has been mostly restricted to the sequence identification of the TLR2 gene in a few fish species (zebrafish, japanese flounder, fugu and catfish) with limited studies on the basal and inducible expression levels of TLR2 transcripts (17-20). Although these studies demonstrate the presence of a TLR2 orthologue, the role of the TLR2 receptor in the recognition of ligands from Gram-positive bacteria has not been studied in fish.

Teleost fish possess orthologs of the different mammalian TLR families and a further two additional fish-specific TLR family members (17, 21). So far, only for the orthologs of TLR3 in zebrafish and rainbow trout (22, 23) and TLR5 in rainbow trout (24) proof for functional analogy to the mammalian counterparts has been provided. In fact, clear differences between fish and mammalian vertebrates in the recognition and response to certain PAMPs have been observed. Cells from teleost fish require much higher doses (in the order of μg/ml) of lipolysacharides (LPS) from Gram-negative bacteria than human cells (ng/ml) for activation (25, 26). In teleost fish, LPS signals via a TLR4-independent manner (27) and, in fact, it was hypothesized that the TLR4 genes in zebrafish are paralogous rather than orthologous to human TLR4 (28). The activation of fish cells by a prototypical TLR ligand such as LPS thus provides no evidence for a direct relationship between ligand, the corresponding receptor and the resulting immune response and classification of TLR proteins solely based on sequence homology may lead to false presumptions on functional conservation (28). In addition, although in mammalian vertebrates cytokine and radical production have been extensively used as markers of TLR(2) activation (29, 30), differences in the activity of certain cytokines in fish, such as TNF-α (31, 32), indicate that TLR activation and subsequent cytokine induction do not necessarily have homologous functions in mammalian and teleost vertebrates. This led us to carefully examine the ligands for TLR2 and activation of cytokines and nitrogen and oxygen radicals downstream of TLR2 activation in fish.

In mammalian vertebrates, TLR2-mediated recognition of PAMPs from Gram-positive bacteria such as lipoteichoic acid (LTA) or peptidoglycan (PGN) results in recruitment of a set of TIR domain-containing adaptor proteins such as MyD88. These interactions trigger downstream cascades leading to the activation of nuclear factor-kB (NF-κB) and mitogen-activated protein kinases (MAPKs), which control induction of inflammatory genes like IL-1β, TNF-α and iNOS and control the activation of antimicrobial host defense mechanisms such as production of reactive oxygen (ROS) and nitrogen (NO) species (29, 30, 33, 34). In general, in teleost fish, the intracellular components acting downstream of TLR receptors are highly conserved, with orthologous sets of genes in mammalian vertebrates and teleosts (35). The extracellular components of receptors such as TLRs seem to be more divergent (36). We analyzed the TLR2 receptor sequence of common carp (Teleostei, Cyprinidae) and found a high degree of conservation of the position of leucine residues critical for ligand recognition. Transfection of human cells (HEK 293) and carp macrophages with the carp TLR2 receptor confirmed activation by the

prototypical ligands from Gram-positive bacteria LTA, PGN and Pam_3CSK_4, but not MALP-2. Activation of carp macrophages indicated that fish cells require high concentrations of ligands from Gram-positive bacteria (LTA, PGN) for activation and signal transduction, react less strongly (Pam_3CSK_4) or do not react at all (MALP-2). However, LTA and PGN from *S. aureus* did regulate carp TLR2 gene expression and enhanced TLR2 mRNA stability. Furthermore, our results suggest that H_2O_2 radicals *via* MAPK-p38 activation play an indispensable role in the regulation of TLR2 gene expression in carp macrophages. Further studies may confirm that our observations also apply to other teleost fish species.

MATERIAL AND METHODS

Animals

European common carp (*Cyprinus carpio carpio* L.) were reared in the central fish facility at Wageningen University, The Netherlands at 23 °C in recirculating UV-treated tap water and fed pelleted dry food (Trouvit, Nutreco) daily. R3xR8 carp are the hybrid offspring of a cross between fish of Hungarian origin (R8 strain) and of Polish origin (R3 strain) (37). Carp were between 9 and 11 months old. All studies were performed with approval from the animal experimental committee of Wageningen University.

Stimuli and inhibitors

Purified lipotheichoic acid from *Staphylococcus aureus* (tlrl-psLTA), soluble secreted peptidoglycan from *Staphylococcus aureus* (tlrl-ssPGN), synthethic tri-palmitoylated lipopetide Pam_3CSK_4 (tlrl-pms) and ultrapure lipopolysaccharide from *Porphyromonas gingivalis* (tlrl-pgLPS) were purchased from InvivoGen (Cayla SAS, France). Synthetic MALP-2, S-[2,3-bis(Palmityloxy)-(2R)-propyl-cisteinyl-GNNDESNISFKEK] was purchased from Alexis Biochemicals (Axxora, Germany). Lipopolysaccharide from *Escherichia coli* (L2880), muramyl dipeptide and polyinosinic polycytidiylic acid (poly-I:C) were purchased from Sigma-Aldrich (St. Louis, MO USA). NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC), xanthine oxidase inhibitor allopurinol, mitochondrial chain I inhibitor rotenone, NAD(P)H oxidase inhibitor diphenyleneiodonium chloride (DPI), superoxide dismutase (SOD) and catalase were purchased from Sigma-Aldrich. MAPK-p38 inhibitor SB208530 was purchased from Calbiochem (Merck Chemicals Ltd,UK). Phosphatidylinositol specific phospholipase C (PI-PLC) from *Bacillus cereus* was purchased from Sigma-Aldrich (St. Louis, MO USA).

Amplification of carp TLR2WT and TLR2ΔTIR cDNA

Oligonucleotide primers for carp TLR2 were designed based on known partial fish TLR2 sequences. cDNA from macrophages stimulated with LTA for 6h was used as template for PCR or nested PCR. The 5´and 3´ends of TLR2 were amplified using gene-specific primers (Forward TLR2 & Reverse TLR2, Table I) by 5´and 3´rapid amplification of cDNA ends (RACE) (38) using the Gene Racer™ RACE Ready cDNA kit (Invitrogen, Breda, The

Netherlands) according to the manufacturer´s protocol. Gene-specific primer (TLR2 Fw) was used in combination with the Gene Racer primers to amplify first strand of cDNA (Table I). A second round with gene-specific primers (TLR2Fw, TLR2Rv) was performed to obtain the carp wild-type TLR2 (TLR2 WT, 2367bp) or TIR-domain truncated TLR2 (TLR2Fw, TLR2Rvnested) (TLR2ΔTIR, 2100bp). PCR reactions were performed in *Taq* buffer, using 1U *Taq* polymerase (Promega, Leiden, The Netherlands) supplemented with $MgCl_2$ (1.5 mM), dNTPs (200 μM) and primers (400 nM each) in a total volume of 50 μl. PCR and nested PCR were carried out under the following conditions: one cycle 4 min at 96 °C; followed by 35 cycles of 30 sec at 96 °C, 30 sec at 55 °C and 2 min at 72 °C; and final extension for 7 min at 72 °C, using a GeneAmp PCR system 9700 (PE Applied Biosystems, Foster City, CA). Products amplified by PCR, nested PCR or RACE-PCR were ligated and cloned in JM-109 cells using the pGEM-Teasy kit (Promega) according to the manufacturer´s protocol. From each product both strands of eight clones were sequenced, using the ABI prismBigDye Terminator Cycle Sequencing Ready Reaction kit and analysed using 3730 DNA analyser.

Bioinformatics

Nucleotide sequence was translated using the ExPASy translate tool (http://us.expasy.org/tools/dna.html) and aligned with Clustal W (http://www.ebi.ac.uk/clustalw). The signal peptide cleavage site and the transmembrane region was predicted by using the SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) and the TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/) servers, respectively. Identification of leucine-rich repeats (LRRs) within carp TLR2 were predicted using the method previously described (39). First, LRRs were predicted using PFAM (http://pfam.sanger.ac.uk/) and SMART (http://smart.embl-heidelberg.de/) servers. Second, LRRs candidates, that could not be recognized by PFAM, were identified by multiple sequence alignments with TLR2 from other species including human, mouse and zebrafish. Third, protein secondary structure predictions of the LRR candidate were evaluated by Proteus (http://wks16338.biology.ualberta.ca/proteus/) server. Post-translational modifications were predicted using the NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/) server.

TLR2 WT-GFP and TLR2ΔTIR-GFP expression plasmids

The vivid color™pcDNA™6.2/C-EmGFP-GW/TOPO® (Invitrogen, catalog no. K359-20) expression vector combined with TOPO®cloning was used to fuse TLR2 WT or TLR2ΔTIR to EmGFP at the C-terminal end. Isolation of highly pure plasmid DNA suitable for transfection was performed using S.N.A.P.™ Midi Prep Kit (Invitrogen, catalog no. K1910-01) according to the manufacturer´s protocol. C-terminal fluorescent-tagged protein could be visualized using confocal microscopy.

Transient transfection of HEK 293 cells

Human embryonic kidney 293 (HEK 293) cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 50 U/ml penicillin G (Sigma-Aldrich)

and 50 µg/ml streptomycin sulphate (Sigma-Aldrich). Two days before transfection, HEK 293 cells were seeded into tissue culture flasks to reach 80-90% confluence at the day of transfection. For transient transfection, 2.5 µg of the carp TLR2 WT-GFP or TLR2ΔTIR-GFP constructs was transfected into HEK 293 by nucleoporation using nucleofactor™ solution V and program A-23 (Lonza Cologne AG, Germany) according to the manufacturer´s instructions. The transfection efficiency, assessed by flow cytometry, was on average 70%. Forty-eight hours after transfection, cells were trypsinized (0.5% trypsin, GIBCO), counted using Trypan blue exclusion and plated overnight at a concentration 6 x 10^5 cells/ well in a 24-well tissue culture plate. The next day, cells were stimulated with (optimized concentrations of) 50 µg/ml LTA, 50 µg/ml PGN, 10 µg/ml Pam_3CSK_4, 10 µg/ml MALP-2, 50 µg/ml *Pg*LPS , 50 µg/ml pIC, 50 µg/ml *Ec*LPS for 5 min or left untreated as control. Cells were lysed for evaluation of phospho-p38 activity by Western blot.

Macrophage cell culture

Head kidney-derived macrophages, considered the fish equivalent of bone marrow-derived macrophages, were prepared as previously described (40, 41). Briefly, carp head-kidneys were gently passed through a 100 µm sterile nylon mesh (BD Biosciences, Breda, The Netherlands) and rinsed with homogenization buffer [incomplete-NMGFL15 medium containing 50 U/ml penicillin G, 50 µg/ml streptomycin sulphate, and 20 U/ml heparin (Leo Pharmaceutical, Weesp, The Netherlands)]. Cell suspensions were layered on 51% (1,07 $g.cm^{-3}$) Percoll (Amersham Biosciences, Uppsala, Sweden) and centrifuged at 450 *g* for 25 min at 4 °C with the brake disengaged. Cells at the interphase were removed and washed twice in incomplete NMGFL-15 medium. Cell cultures were initiated by seeding 1.75 x 10^7 head kidney leukocytes in a 75 cm^2 culture flask containing 20 ml of complete NMGFL-15 medium [incomplete-NMGFL-15 medium supplemented with 5% heat-inactivated pooled carp serum and 10% fetal bovine serum]. Head kidney-derived macrophages, named macrophages throughout the manuscript, were harvested after 6 days of incubation at 27 °C by placing the flasks on ice for 10 min prior to gentle scraping.

Transient transfection of carp macrophages

For transient transfection, 2.5 µg of the carp TLR2 WT-GFP or TLR2ΔTIR-GFP constructs was transfected into carp macrophages by nucleoporation using nucleofactor™ Human Macrophage Solution and program Y-001 (Lonza Cologne AG, Germany) according to the manufacturer´s instructions and placed into 48-well plate at a concentration 1 x 10^6 cells/ well. The transfection efficiency, assessed by flow cytometry, was on average 25%. After 24h incubation, the medium was replaced and macrophages were stimulated for 6h or 9h with (optimized concentrations of) 50 µg/ml LTA, 50 µg/ml PGN, 10 µg/ml Pam_3CSK_4, 10 µg/ml MALP-2 or left untreated as control. Cells were lysed for evaluation of gene expression by real-time quantitative PCR.

Confocal laser scanning microscopy

Cytospin microscope slides of carp macrophages were made by fixing cells in 100% alcohol

and 37% formaldehyde (10:1). HEK 293 cells were grown in 6 well glass bottom culture plate (MatTek,USA) fixed in 100% alcohol and 37% formaldehyde (10:1). Sections were embedded in Vectashield containing propidium iodide (Vector Laboratories) and examined with a Zeiss LSM-510 laser scanning microscope. Green fluorescent signal (rhodamine or green-fluorescent protein) was excited with a 488 nm argon laser and detected using a band-pass filter (505-530 nm) and red-fluorescent signal (propidium iodide) was excited with a 543 nm helium-neon laser and detected using a long-pass filter (560 nm).

Magnetic activated cell sorting (MACS)

Macrophage-enriched fractions of head kidney leukocytes were obtained essentially as previously described (42). Cell suspensions were layered on a discontinous Percoll gradient (1.020, 1.060, 1.070 and 1.083 g cm^{-3}) and centrifuged 30 min at 800 *g* with the brake disengaged. Cells at 1.070 and 1.083 g cm^{-3} were collected, pooled and washed twice with cRPMI [RPMI 1640 adjusted to carp osmolarity 280 $mOsmkg^{-1}$]. Relative cell populations were found to be similar as previously described: 1.070 g cm^{-3} interphase consisted of macrophages (65%) with granulocytes, small macrophages and lymphocytes (35%) and 1.083 g cm^{-3} interphase consisted of neutrophilic granulocytes (85%) with macrophages (15%). The monoclonal antibody TCL-BE8 (1:50) (43) was used to separate neutrophilic granulocytes from macrophages by MACS. After incubation for 30 min with TCL-BE8 on ice, the leukocyte suspension was washed twice with cRPMI and incubated with phycoerythrin (PE)-conjugated goat anti-mouse (1:50; DAKO, Glostrup, Denmark) 30 min on ice. After washing twice, the total cell number was determined with a Bürker counting chamber, and 10 µl of magnetic beads (anti-PE Microbeads, Miltenyi Biotec, GmbH, Germany) was added per $1x10^8$ cells. After incubation for 15 min at 4 °C, cells were washed twice and resuspended in cRPMI. The magnetic separation was performed on LS-MidiMACS Columns (Mitenyi Biotec) according to the manufacturer's instructions. The purity of the TCL-$BE8^+$ (neutrophilic granulocyte-enriched fraction; > 90%) and TCL-$BE8^-$ (macrophage-enriched fraction; < 10%) fractions was confirmed by flow cytometric analysis using a FACScan® flow cytometer (Becton Dickinson, Mountain View, CA, USA). TCL-$BE8^+$ and TCL-$BE8^-$ were washed in cRPMI and resuspended in 1 ml of $cRPMI^{++}$ [cRPMI medium supplemented with 1.5% pooled carp serum, 290 µg/mL glutamine (Cambrex), 50 U/ml penicillin G and 50 µg/ml streptomycin sulphate].

Flow cytometric measurement of ROS production

Intracellular ROS levels were evaluated by FACScan® flow cytometer using the redox-sensitive dye dihydrorhodamine 123 (DHR) (44, 45) at 0.25 µg/ml (Sigma, D1054). Head-kidney derived macrophages (5×10^5) were resuspended in 100 µL rich-NMGFL-15 medium [incomplete-NMGFL-15 medium supplemented with 2.5% heat-inactivated pooled carp serum and 5% fetal bovine serum] and stimulated with PMA (0.05 µg/ml; Sigma) or left untreated as controls. Concomitantly, cells were also stimulated with LTA or PGN. DHR was added to all samples, and cells incubated for 1 h at 27 °C. Cells were resuspended by pipetting, transferred to flow cytometer tubes and propidium iodide (PI; 0.1 mg/ml; Sigma)

was added to each sample to detect and gate out PI^+ cells. For all cytometric measurements the same settings were used (FS 350 volt, gain 2; SS 700 volt, gain 10; FL1 600 volt; FL2 750 volt; FL3 675 volt; FL4 660 volt). The baseline offset was on and the FS discriminator was set at 50. Per sample, 10^4 events were measured by flow cytometer.

Nitrite production

Macrophages were stimulated with LTA (50 µg/ml), PGN (50 µg/ml), or left untreated as control, and incubated at 27°C for 18h. Nitrite production was measured essentially as described before (46): to 75 µl of cell culture supernatant, 100 µl of 1% sulfanilamide in 2.5% (v/v) phosphoric acid and 100 µl of 0.1% (w/v) *N*-naphthyl-ethylenediamine in 2.5% (v/v) phosphoric acid were added in a 96-well flat-bottom plate. The absorbance was read at 540 nm (with 690 nm as a reference) and nitrite concentration (µM) was calculated by comparison with a sodium nitrite standard curve.

Gene expression profiling of LTA and PGN stimulated macrophages

Head kidney derived-macrophages (5 x 10^5) in 100 µl rich-NMGFL-15 were seeded in 96-well flat-bottom culture plates and stimulated with LTA, PGN, Pam_3CSK_4 or left untreated as control, and incubated for the indicated time points at 27 °C. A number of 3-5 x 10^6 cells (combination of 6-10 wells) per time point, per treatment, were lysed and pooled for RNA isolation. RNA was isolated using the RNeasy Mini Kit (Qiagen, Leusden, The Netherlands) including the accompanying DNase I treatment on the columns, according to the manufacturers´ protocol. Final elution was performed with 30 µl nuclease-free water. RNA concentrations were measured by spectrophotometry (Nanodrop, Thermo scientific, Breda, The Netherlands) and 1 µl was analysed on a 1 % agarose gel to check the RNA integrity. RNA was stored at -80°C until further use. Prior to cDNA synthesis, a second DN*ase* treatment was performed using DN*ase* I, Amplification Grade (Invitrogen). Briefly, 1 µg of RNA from each sample was combined with 10X DN*ase* reaction buffer and 1 U DN*ase* I, mixed and incubated at RT for 15 min, followed by inactivation of DN*ase* I by adding 1 µl of 25 µM EDTA. Synthesis of cDNA was performed with Invitrogen's SuperScript™ III First Strand Synthesis Systems for RT-PCR Systems, according to the manufacturer's instructions. Briefly, DN*ase* I-treated RNA samples were mixed with 5x first strand buffer, 300 ng random primers, 10 µM dNTPs, 0.1 M DTT, 10 U RN*ase* inhibitor, and 200 U SuperScript III Reverse Transcriptase up to a final volume of 20 µl. The mixture was incubated at 37 °C for 60 min followed by an inactivation step at 70 °C for 15 min. A non-reverse transcriptase control was included for each sample. cDNA samples were further diluted 50 times in nuclease-free water before use as template in real-time PCR experiments.

Real time quantitative PCR (RT-qPCR) was performed in a 72-well Rotor-Gene™ 2000 (Corbett Research, Mortlake, Sydney, Australia) with the Brilliant® SYBR® Green QPCR (Stratagene, La Jolla, CA, USA) as detection chemistry. Primers used for RT-qPCR (Table I) were designed with Primer Express software. IL1-β and TNF-α primer sets were designed to amplify all known isoforms for each gene.

Master-mix for each PCR run was prepared as follows: 0.32 µl of water, 0.84 µl of each primer (5 µM), 7 µl Master SYBR Green I mix. To 5 µL of diluted cDNA, 9 µl of master mixed was added in a 0.1 ml tube. Following amplification program was used: one denaturation step of 15 min at 95 °C; followed by 40 cycles of RT-qPCR with three-step amplification (15 s at 95 °C for denaturation, 30 s at 60 °C for annealing and 30 s at 72 °C for elongation) and a final holding step of 1 min at 60 °C. A melting step was then performed with continuous fluorescence acquisition starting at 60 °C with a rate of 1 °C/5 s up to 99 °C to determine the amplification specificity. In all cases, the amplifications were specific and no amplification was observed in negative controls (non-template control and non-reverse transcriptase control). Fluorescence data from RT-qPCR experiments were analysed using Rotor-Gene version 6.0.21 software and exported to Microsoft Excel. The cycle threshold C_t for each sample and the reaction efficiencies (E) for each primer set were obtained upon Comparative Quantitation Analysis from the Rotor-Gene version 6.0.21 software. Briefly, the E for each primer set was recorded per sample and an average E (E_A) was then calculated for each primer set. The relative expression ratio (R) of a target gene was calculated based on the E_A and the C_t deviation of sample versus control, and expressed in comparison to a reference gene (47, 48).

Table I: Primer sequences, gene accession numbers, RT-qPCR melting temperatures and efficiencies

Primer	Sequence (5'-3')	GenBank Accession No.	Melting Temperature (℃)	Efficiency
RACE				
Forward TLR2	CAA CAC TCT TAA TGT GAG CCA GA			
Reverse TLR2	GCT TTC TGC CAC CAC CCT TGG			
cDNA				
TLR2 Fw	AGGATGGAATTCTTGGGAAGA			
TLR2 Rv	ACATTCATCTCTCTGTAGAGCAGCTCTCAGTTTGC			
TLR2 Rvnested	AGTCCGATGGCTCTTCTCA			
RT-qPCR				
IL-1β Fw	AAGGAGGCCAGTGGCTCTGT	AJ245635	79.0	1.74
IL-1β Rv	CCTGAAGAAGAGGAGGAGGCTGTCA			
IL-11 Fw	CAGCAGCACAGCTCAGTACCA	AJ632159	79.3	1.75
IL-11 Rv	AGCCTCTGCTCGGGTCATCT			
iNOS Fw	AACAGGTCTGAAAGGGAATCCA	AJ242906	77.8	1.73
iNOS Rv	CATTATCTCTCATGTCCAGAGTCTCTTCT			
TNF-α Fw	GCTGTCTGCTTCACGCTCAA	AJ311800-01	77.5	1.77
TNF-α Rv	CCTTGGAAGTGACATTTGCTTTT			
IL12-p35 Fw	TGCTTCTCTGTCTCTGTGATGGA	AJ580354	77.5	1.75
IL12-p35 Rv	CACAGCTGCAGTCGTTCTTGA			
IL12-p40a Fw	GAGCGCATCAACCTGACCAT	AJ621425	77.2	1.76
IL12-p40a Rv	AGGATCGTGGATATGTGACCTCTAC			
TLR2 Fw	TCAA CA+C TCT TAA TG+T GAG CCA[a]	FJ858800	78.0	1.82
TLR2 Rv	TGT G+CT GGA AA+G GTT CAG AAA[a]			
40S Fw	CCGTGGGTGACATCGTTACA	AB012087	78.5	1.77
40S Rv	TCAGGACATTGAACCTCACTGTCT			

[a] "+" is before the nucleic acid in which locked nucleic acid (lna) bond was placed

Western blot analysis

Cells were resuspended by pippeting and transferred to pre-cooled eppendorf tubes. Cells were washed twice in ice-cold PBS, lysed on ice with lysis solution [0.5% Triton X-100, 20 mM Tris, 100 mM NaCl, 1 mM EDTA, 50 mM NaF (Sigma), 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma)], homogenized with a syringe and incubated 10 min on ice. Cell lysates were centrifuged at 14000 rpm for 10 min at 4 °C. Supernatant was collected and total protein content was determined by the Bradford method. Samples (20-25 µg) were boiled at 96 °C for 10 min with loading buffer containing β-mercaptoethanol and separated by 10% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes (Protrans, Schleicher & Schuell, Bioscience GmbH). Membranes were blocked in 3% BSA in TBS (10 mM Tris, 150 mM NaCl, pH 7.5) for 1 h at room temperature and then incubated with primary antibody overnight at 4 °C in 3% BSA in TBS. Following antibodies reactive to both humans and carp were used: rabbit IgG anti-phospho-p38 (1:1000, Thr180/Tyr182, BioCat GmBh, Heidelberg, Germany) and rabbit IgG anti-β-tubulin (1:1000, Abcam, Cambridge, UK). Membranes were then incubated with goat-anti-rabbit HRP-conjugated (1:1000, Dako) in 10% milk powder in TBS for 1 h at room temperature. Between each incubation step, membranes were washed twice with TBS-Tween/Triton (TBS, 0.05% (v/v) Tween 20, 0.2% (v/v) Triton X-100) and once with TBS, for 10 min at room temperature. Signal was detected by development with a chemoluminescence kit (Amersham) according to the manufacturer's protocol and visualized by the use of Lumni-fil chemiluminescent Detection Film (Roche, Woerden, The Netherlands). The blots were scanned and the intensity profile (between 0 and 255) of each lane was determined over a horizontal line on a grayscale image using the analysis FIVE (Olympus Nederland BV) program.

Protozoan-derived PAMPs

Phosphatidylinositol-specific phosphalipase C (PI-PLC) from *Bacillus cereus* was resuspended in Tris buffer (10 mM Tris-HCl, 144 mM NaCl, 0.05% BSA, pH=7.4) according to the manufacturers' protocol. The protozoan carp parasite *Trypanoplasma borreli* (49) was incubated for 30 min at 30 ºC with 30% Tris-buffer or with 1 unit PI-PLC (in 30% Tris-buffer) up to a total of 600 µL in incomplete NMGFL-15 medium. Samples were centrifuged at 800*g* for 10 min. Supernatants were collected and filter-sterilized (0,22 µm Millex-GV, Millipore, Ireland) to avoid contamination with parasites. Pellets from PI-PLC-treated were collected and resuspended in 600 µL incomplete NMGFL-15 medium. Carp macrophages were stimulated with 25 µL of each fraction.

Statistical Analysis

Transformed values (ln) were used for statistical analysis in SPSS software (version 15.0). Homogeneity of variance was analyzed using the Levene's test. Significant differences ($P \leq 0.05$) between a treatment (stimulated cells) and the control group (unstimulated cells) were determined by a one-way ANOVA followed by a Dunnett T-test. Significant differences between treatments ($P \leq 0.05$) were determined by one-way ANOVA followed

by Bonferroni test. In case of unequal variances between treatments, the one-way ANOVA was followed by a Games–Howell test.

RESULTS

Carp TLR2 ectodomain contains the critical residues for ligand recognition

Full-length cDNA of the carp TLR2 gene was cloned (GenBank Accession no. FJ858800, http;//www.ncbi.nlm.nih.gov/Genbank) encoding for a TLR2 protein of 788 amino acids. The carp TLR2 protein is composed of an extracellular, transmembrane and intracellular domain. The ability of carp TLR2 to recognize prototypical TLR2 ligands and to trigger characteristic downstream cascades was evaluated by comparative sequence analysis. The extracellular domain of carp TLR2 has 21 leucine rich repeats (LRR), whereas human and mouse TLR2 have 20 LRRs (excluding the LRRs at the N-terminal and C-terminal region) (Fig. 1A).

A

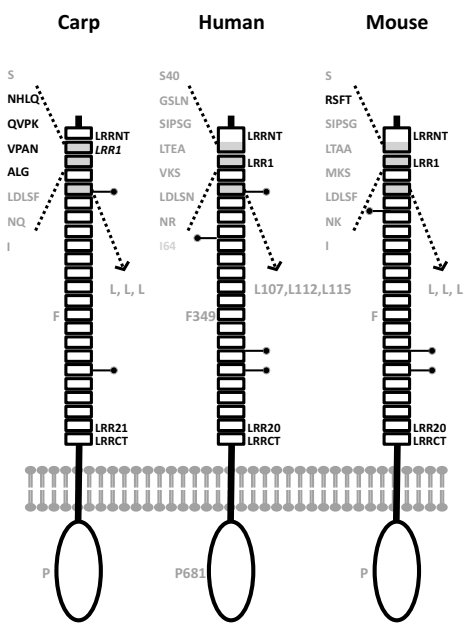

Figure 1. Schematic representation of carp, human and mouse TLR2 on the cell membrane. A. All TLR2 molecules have an extracellular, transmembrane and intracellular domain. LRR motifs are indicated by rectangles and the TIR domain is indicated by an ellipse. Human and mouse TLR2 have 20 LRRs, whereas carp TLR2 has 21 LRRs. The additional LRR is located at the N-terminus (*LRR1*) and is an irregular LRR belonging to the 'bacterial' motif. *N*-glycosylation sites are indicated by black circle lollipops. Numbered amino acid residues correspond to the position in the human TLR2 molecule. Regions and amino acid residues shown to be important for ligand recognition, protein secretion or interaction with MyD88 for the human TLR2 molecule are shaded in grey. Conserved amino acid residues in carp or mouse, when compared to human TLR2, are depicted in grey and non-conserved are depicted in black. LRRNT or LRRCT = LRR N-terminal or C-terminal. B. LRR and TIR domain percentage sequence identity between carp and zebrafish, human and mouse TLR2 proteins, determined by Clustal W alignment. GenBank Accession nos: common carp *(Cyprinus carpio*, FJ858800), zebrafish (*Danio rerio*, NP_997977) human (*Homo sapiens*, NP_003255); mouse (*Mus musculus*, NP_036035).

B

		Zebrafish	Human	Mouse
Carp	LRR	78	37	35
	TIR	89	69	67

This additional but irregular LRR at the N-terminal of carp TLR2 belongs to the "bacterial" type and has also been found in the zebrafish TLR2 sequence (39). The extracellular domain of carp TLR2 region containing S40-I64 compassing the LRRNT and LRR1 motifs, shown to be crucial for the recognition of *Staphylococcus aureus* peptidoglycan (50), contained a number of conserved critical LRR. Furthermore, leucine residues at positions 107, 112 and 115 (at LRR3) that are critical in the recognition of diacylated lipoproteins, lipopeptides and *Staphylococcus aureus* peptidoglycan in human TLR2 (51), were conserved. The F349 shown to be involved in the recognition of bacterial lipopetide was also conserved. *N*-glycosylation sites involved in secretion of the N-terminal ectodomain (52) such as N114 (LLR3) and N442 (LRR16) were conserved (Fig. 1A). Thus, despite a relatively low aminoacid sequence identity (<37%) of the carp TLR2 LRR domain to mammalian vertebrate LRR domains (Fig. 1B), the position of the residues critically involved in the recognition of *Staphylococcus aureus* peptidoglycan seems well conserved.

The intracellular (TIR) domain of carp TLR2 showed a much higher sequence identity to the mammalian vertebrate TLR2 sequences than the extracellular LRR domain (Fig. 1B). Proline P681, involved in the interaction with the MyD88 adaptor molecule (53), was conserved in the carp TLR2 sequence. The high sequence identity of the carp TIR domain (>67%) suggests that downstream cascades triggered upon TLR2 activation may be conserved in carp.

LTA, PGN and Pam_3CSK_4, but not MALP-2, are ligands of carp TLR2

HEK 293 cells were used to define ligands for carp TLR2 using MAPK-p38 phosphorylation as a measure for responsiveness (15). HEK 293 cells were transfected with carp TLR2 full-length (TLR2 WT) or with carp TIR-domain truncated TLR2 (TLR2ΔTIR). Both constructs were fused to GFP at the 3′-end which enabled us to investigate the sublocalization of the protein after transfection, by confocal imaging. We could confirm the predominant localization of TLR2 WT on the cell surface (TLR2 WT; Fig. 2). A more diffuse localization was observed after truncation of the TIR domain (TLR2ΔTIR; Fig. 2).

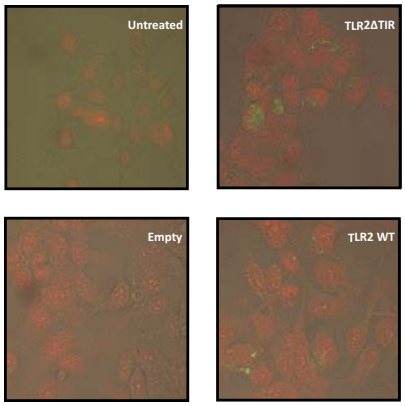

Figure 2. Localization of carp TLR2 contructs in transfected HEK 293 cells. HEK 293 cells were transiently transfected with pTOPO containing full length carp TLR2 (TLR2 WT, 2.5 µg) or TIR-domain truncated TLR2 (TLR2ΔTIR, 2.5 µg) fused to GFP at the 3'-end. Localization of carp TLR2 WT and TLR2ΔTIR was determined by confocal laser microscopy.

MAPK-p38 phosphorylation in transfected HEK 293 cells was used to study carp TLR2 activation by different TLR ligands, using an antibody specific for phosphorylated p38 (P-p38). Stimulation of TLR2ΔTIR transfected HEK 293 cells with known TLR2 ligands did not increase p38 phosphorylation (TLR2ΔTIR; Fig. 3 A, B). This negative control thereby showed the unresponsiveness of the parental HEK 293 cells to TLR2 agonists, confirming that HEK 293 cells themselves do not bear receptors for TLR2 ligands. In contrast, stimulation with LTA, PGN and the synthetic agonist Pam_3CSK_4 (ligand for TLR2/TLR1, (54)) of TLR2 WT transfected HEK 293 resulted in a clear increase of MAPK p38 activity. Neither MALP-2 (ligand for TLR2/TLR6, (12)), nor *Pg*LPS, *Ec* LPS nor only pI:C were able to modulate MAPK p38 activation (TLR2 WT; Fig. 3 A, B). The increase of MAPK-p38 phosphorylation in TLR2 WT transfected and unresponsiveness of TLR2ΔTIR transfected HEK 293 cells to LTA, PGN and Pam_3CSK_4 clearly linked increased MAPK-p38 phosphorylation to the presence of carp TLR2.

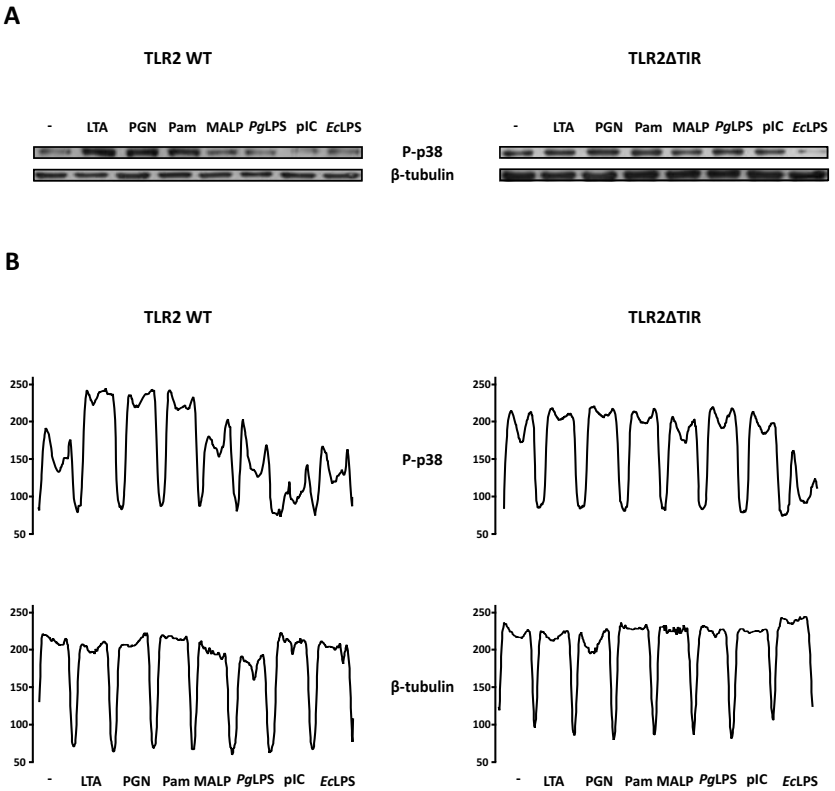

Figure 3. Activation of carp TLR2 by different PAMPs in HEK 293 cells. TLR2 WT and TLR2ΔTIR transfected HEK293 cells were stimulated with LTA (50 μg/ml), PGN (50 μg/ml), Pam_3CSK_4 (Pam_3, 10 μg/ml), MALP-2 (10 μg/ml), *Pg* LPS (50 μg/ml), pIC (50 μg/ml) or *Ec* LPS (50 μg/ml) for 5 min or left untreated as control. A. MAPK-p38 phosphorylation was analyzed by immunoblotting for phospho-p38 (P-p38), while equal loading was confirmed by immunoblotting for β-tubulin. B. Immunoblot intensity profile (between 0 and 255) of TLR2 WT- and TLR2ΔTIR-transfected HEK 293 cells was determined over a horizontal line on each grayscale image. This is one experiment representative of four experiments.

The above-mentioned conservation of the position of the leucine residues critical for ligand (PGN) recognition in the carp extracellular LRR domain (Fig. 1) suggested that carp TLR2 should be able to bind TLR2 ligands. Transfection of HEK 293 cells with TLR2 WT corroborated the ability of carp TLR2 to bind the prototypical mammalian vertebrate TLR2 ligands LTA, PGN and Pam_3CSK_4, but not MALP-2.

Activation of carp macrophages by TLR2 ligands

Basal TLR2 gene expression was measured in different cell types and shown to be most ubiquitous in myeloid-derived cells and highest in macrophages (data not shown). In mammalian vertebrates, TLR activation results in downstream gene expression of immune modulators including several cytokines, chemokines and radicals such as nitric oxide. This immune profile is often used as marker for TLR(2) activation. We tested the ability of LTA, PGN, Pam_3CSK_4 and MALP-2 to induce an immune gene expression profile in carp macrophages. Pam_3CSK_4 modulated immune gene expression to a minor extent whereas MALP-2 clearly did not modulate immune gene expression at any concentration (1-10 µg/ml) or time point (6-18 h) analyzed.

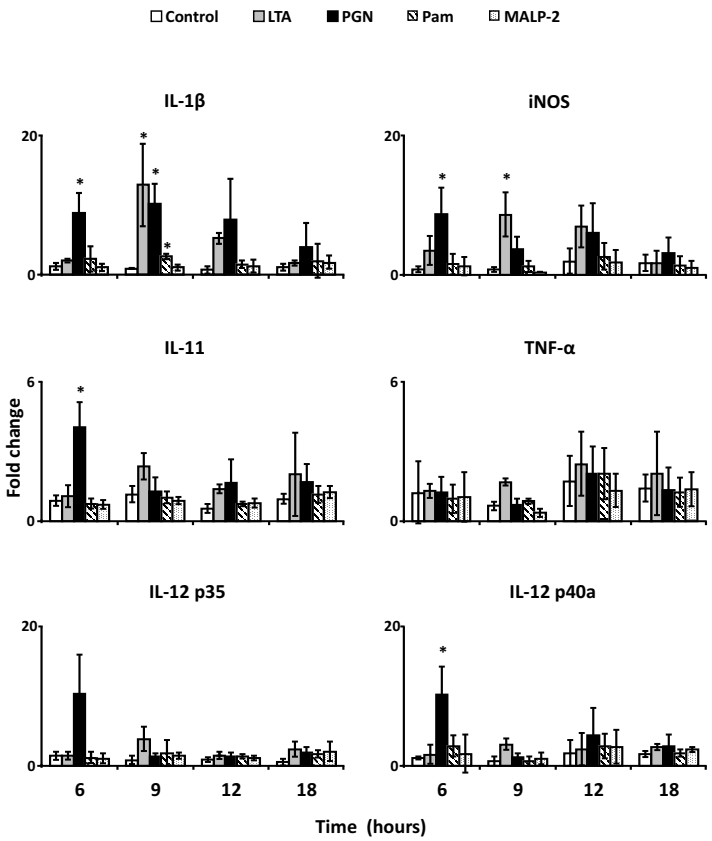

Figure 4. Kinetics of immune gene expression in carp macrophages after stimulation with different PAMPs. Real-time quantitative PCR analysis of gene expression in carp macrophages after stimulation for 6, 9, 12 and 18h with LTA (50 µg/ml), PGN (50 µg/ml), Pam_3CSK_4 (Pam_3, 10 µg/ml), MALP-2 (10 µg/ml) or left untreated as control. mRNA levels of IL-1β, iNOS, IL-11, TNFα, IL12p35 and IL12p40 relative to the house keeping 40S ribosomal protein gene level are expressed as fold change relative to unstimulated cells at 0h. Bars show averages ± SD of n=3 fish. Symbol '*' represents a significant ($P \leq 0.05$) difference compared to unstimulated cells (control group). Note the differences in scale of the Y axes.

In contrast, both LTA and PGN modulated immune gene expression in carp macrophages, albeit at high concentrations of 50 µg/ml. Both the magnitude and the kinetics of the response were different between the two stimulants, with PGN consistently inducing a higher response than LTA. The kinetics of downstream activation of carp IL-1β, iNOS, IL-11, IL-12 p35 (P = 0.065) and IL12-p40 indicated highest gene expression at 6h following PGN-stimulation, whereas LTA stimulation induced highest gene expression at 9h (Fig. 4). Our results show that the TLR2 ligands LTA, PGN and Pam_3CSK_4 (to a minor extent) but not MALP-2, stimulated downstream signaling pathways with ligand-specific kinetics profiles in carp macrophages.

Higher activation of carp macrophages by LTA, PGN and Pam_3CSK_4, but not MALP-2 after overexpression of TLR2

To verify if macrophage activation by LTA and PGN would be TLR2-mediated, we overexpressed TLR2 in carp macrophages. The expression of both TLR2WT and TLR2ΔTIR constructs after transfection of carp macrophages were confirmed by western blot (data not shown). Stimulation of macrophages overexpressing TLR2ΔTIR was used as the negative control (TLR2ΔTIR Fig. 5). Carp macrophages overexpressing TLR2 WT could be further activated with PGN and LTA and to a minor extent with Pam_3CSK_4, but not with MALP-2 (TLR2 WT; Fig. 5). Again, gene expression (IL-1β, iNOS) was highest at 6h, following PGN-stimulation, and highest at 9h after LTA stimulation.

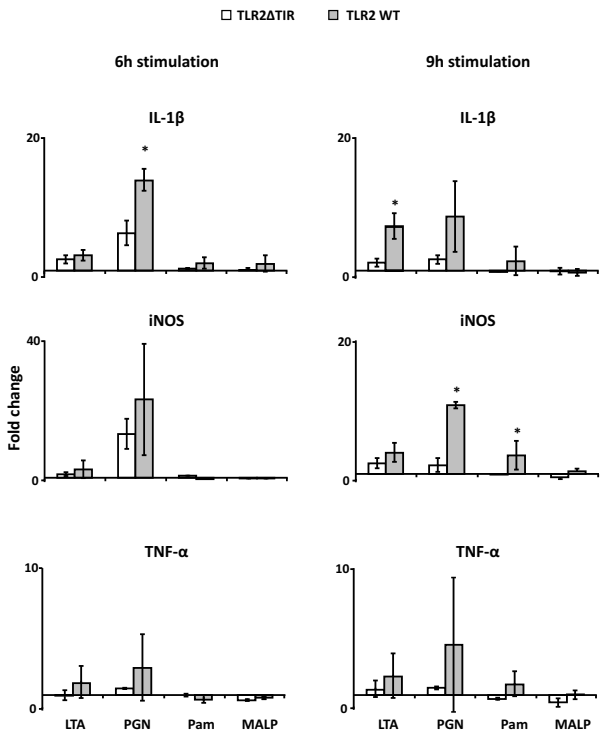

Figure 5. Overexpression of TLR2 in carp macrophages. TLR2 WT and TLR2ΔTIR transfected carp macrophages were stimulated with LTA (50 µg/ml), PGN (50 µg/ml), Pam_3CSK_4 (Pam_3, 10 µg/ml), MALP-2 (10 µg/ml) for 6 and 9h or left untreated as control. mRNA levels of IL-1β, iNOS and TNFα relative to the house keeping 40S ribosomal protein gene level are expressed as fold change relative to unstimulated cells. mRNA levels of IL-11, IL12p35 and IL12p40 after stimulation were also determined but no changes were observed (data not shown). Bars show averages ± SD of n=4 fish. Symbol '*' represents a significant ($P \leq 0.05$) difference compared to TLR2ΔTIR transfected carp cells. Note the differences in scale of the Y axes.

LTA, PGN and protozoan GPI-anchors induce expression and increase mRNA stability of the TLR2 gene in carp macrophages

Prototypical TLR2 ligands LTA and PGN could activate carp macrophages via TLR2 as shown by increased MAPK-p38 phosphorylation in HEK 293 cells and enhanced immune gene expression in TLR2 WT-transfected cells. We subsequently tested if LTA and PGN could modulate the expression of the carp TLR2 gene itself. TLR2 gene expression in carp macrophages was highest at 6h and then declined (Fig. 6A). Stimulation with either LTA or PGN induced a low but significant 1.5-2-fold up-regulation of TLR2 gene expression.

To verify the low but consistent fold up-regulation of TLR2, two strategies were taken. First, additional sources of carp macrophages were examined for TLR2 gene expression. Both macrophage-enriched Percoll fractions of freshly isolated head-kidney leukocytes and macrophage-enriched MACS-sorted leukocytes (TCL-BE8^{-}) showed a consistent but maximum 2-fold up-regulation of expression of the carp TLR2 gene (data not shown). Second, glycosylphosphatidylinositol (GPI) anchors from protozoan parasites of carp, *Trypanoplasma borreli* (Kinetoplastida), were examined as TLR2 ligands. In mammalian vertebrates, TLR2 recognizes GPI-anchors from protozoan parasites (8). Macrophages stimulated with supernatant from PI-PLC-treated parasites as source of GPI-anchors showed a dose-dependent up-regulation of TLR2 gene expression (Fig. 6B). Macrophages stimulated with non-treated parasites or the resultant pellet did not show up-regulation of TLR2 gene expression (negative controls).

Stimulation with LTA more than stimulation with PGN stabilized carp TLR2 mRNA (increased mRNA half-life: Fig. 6B). Whereas IL-1β and iNOS mRNA (control genes) was also stabilized by LTA, this was not the case for stimulation by PGN. Using an inhibitor of MAPK-p38, we also studied the post-transcriptional effect of MAPK-p38 phosphorylation on the mRNA stability of the carp TLR2 gene, again including IL-1β and iNOS as control genes. Inhibition of MAPK-p38 destabilized all three gene transcripts. Our results suggest that the TLR2 ligands LTA and PGN, but also the signaling molecule MAPK-p38, lead to increased mRNA stability of the carp TLR2 gene. Increased mRNA stability may lead to an increase in the amount of TLR2 protein induced after ligand binding.

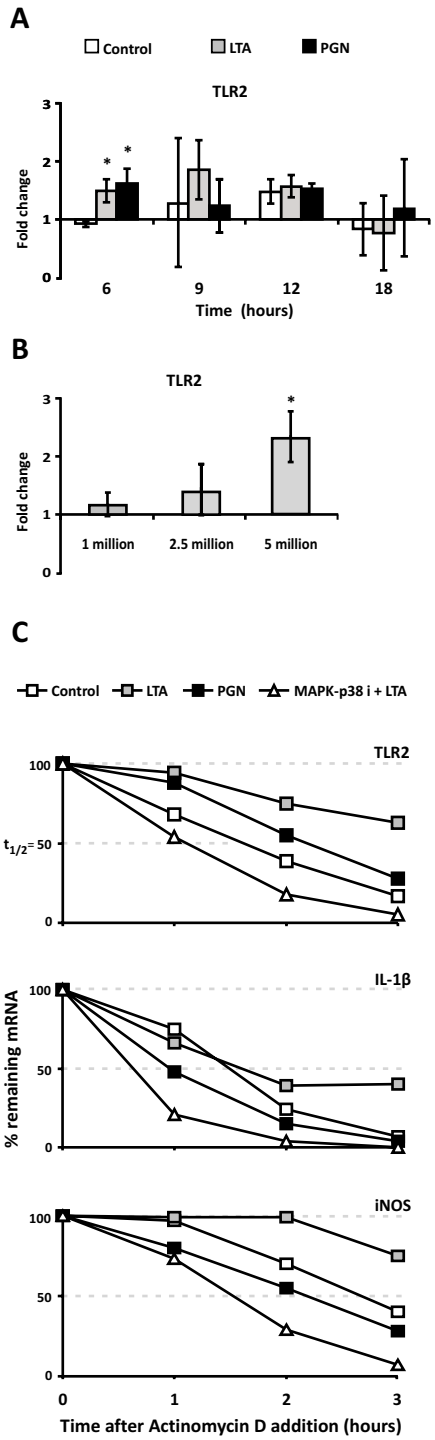

Figure 6. TLR2 gene expression and mRNA stability after stimulation with TLR2 ligands. A. Kinetics of TLR2 gene expression in carp macrophages after stimulation for 6, 9, 12 and 18h with LTA (50 µg/ml), PGN (50 µg/ml) or left untreated as control. Real-time quantitative PCR analysis of gene expression in carp macrophages. mRNA levels of TLR2 relative to the house keeping 40S ribosomal protein gene level are expressed as fold change relative to unstimulated cells at 0h. Bars show averages ± SD of n=3 fish. Symbol '*' represents a significant ($P \leq 0.05$) difference compared to unstimulated cells (control group). B. TLR2 gene expression in carp macrophages after stimulation for 6h with supernatants from PI-PLC-treated *Trypanoplasma borreli* parasites. mRNA levels of TLR2 relative to the house keeping 40S ribosomal protein gene level are expressed as fold change relative to unstimulated cells. Bars show averages ± SD of n=4 fish. Symbol '*' represents a significant ($P \leq 0.05$) difference compared to unstimulated cells (control group). C. TLR2 mRNA stability. TLR2 gene transcription in carp macrophages after stimulation for 6h with LTA (50 µg/ml) or PGN (50 µg/ml) or pre-incubated for 30 min with MAPK-p38 inhibitor (SB203530, 25 µM) followed by stimulation for 6h with LTA (50 µg/ml). At 6h cells were treated with RNA synthesis inhibitor (Actinomycin D, 5 µg/ml) and collected at the time points indicated. mRNA levels of TLR2, IL-1β and iNOS, corrected for differences in mRNA levels of the house keeping 40S ribosomal protein gene, are expressed as % remaining mRNA (ratio mRNA at indicated time point relative to mRNA of the same gene prior to the addition of Actinomycin D). Graphs do not reflect differences in gene transcription between treatments before the addition of Actinomycin D. This is one experiment representative of three experiments. Exponential functions fitting the TLR2 mRNA levels for each treatment were used to estimate half-time ($t_{1/2}$) in hours. Estimated half-time of TLR2 gene transcripts were $t_{1/2}$= 1.18 h for unstimulated cells, and $t_{1/2}$= 4.30 h (LTA), $t_{1/2}$= 1.62 h (PGN) and $t_{1/2}$= 0.69 h for MAPK-p38/LTA- stimulated macrophages.

PGN induces MAPK-p38 activation and radical production in carp macrophages

Stimulation of carp macrophages with LTA and PGN resulted in higher phosphorylation of MAPK-p38 in carp macrophages within 5 min (Fig. 7A). The signal was stronger in PGN- than in LTA-stimulated carp macrophages. The MAPK-p38 pathway is known to be redox-sensitive in mammalian vertebrates (55). PGN, but not LTA, induced the production of nitrogen radicals (NO) in carp macrophages (Fig. 7B). Similarly, oxygen radical (ROS) production by carp macrophages could be induced with PGN (141%) (Fig. 7D), but not with LTA (102%). The ROS production was measured with the DHR 123 probe, which is reduced to the green fluorescent rhodamine 123 (Fig. 7C) under influence of ROS formation (*e.g.* H_2O_2, $HO^{\cdot}$) (45). Treatment of carp macrophages with SOD (which dismutates $O_2^{\cdot-}$ into H_2O_2 and O_2) further increased the ROS production (Fig. 7D) indicating that TLR2-mediated H_2O_2 production can be detected by this method.

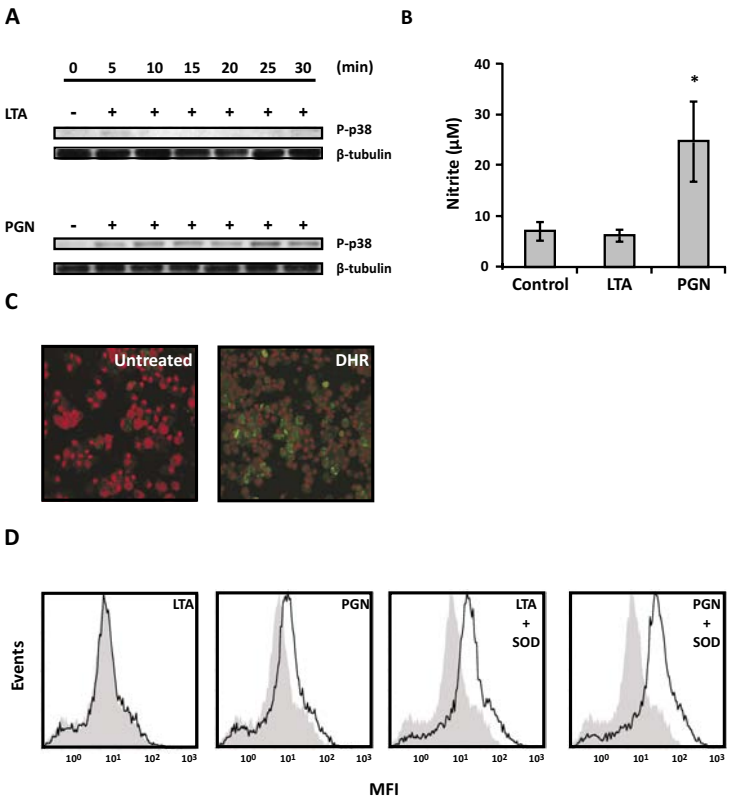

Figure 7. Effect of TLR2 ligands on MAPK-p38 activation and ROS production in carp macrophages. A. MAPK-p38 activation in carp macrophages analyzed by immunoblotting for phospho-p38 (P-p38). Equal loading was confirmed by immunoblotting for β-tubulin. Macrophages were stimulated with LTA (50 µg/ml) or PGN (50 µg/ml) for the time points indicated. B. Nitric oxide production in carp macrophages. Macrophages were incubated with LTA (50 µg/ml) or PGN (50 µg/ml) for 18h. Bars show averages ± SD of n=4 fish. Symbol '*' represents a significant ($P \leq 0.05$) difference compared to unstimulated cells (control group). C. Radical production in carp macrophages analyzed by fluorescence microscopy.Macrophages were incubated for 1h with DHR (0.25 µg/ml) or left untreated as control. D. Radical production in carp macrophages by means of DHR fluorescence intensity. Macrophages were incubated with DHR (0.25 µg/ml) and PMA (0.05 µg/ml) followed by LTA (50 µg/ml) or PGN (50 µg/ml) in the presence or absence of SOD (20 U/ml) for 1h. Grey shaded histograms represent a control sample which corresponds to PMA-stimulated cells. This is one experiment representative of four experiments.

ROS production modulates TLR2 gene expression

PGN induced MAPK-p38 activation, TLR2 gene expression, NO and also ROS production in carp macrophages. To examine the influence of MAPK-p38 activation and ROS production on carp TLR2 gene expression, we used inhibitors of signal transduction and inhibitors of radical production and measured TLR2 gene expression**.** Cell viability (assessed by trypan blue exclusion), TLR2 gene expression and ROS production were not affected by the presence of these inhibitors (data not shown). Signal transduction pathways associated with TLR2 gene regulation were studied by pre-incubation of carp macrophages with inhibitors of NF-κB (PDTC) and MAPK-p38 (SB 208530). TLR2 gene expression, induced by PGN or by LTA (not shown), could be inhibited by the presence of either inhibitor, although the strongest by MAPK-p38 inhibition (Fig. 8A). Radical production, induced by PGN (LTA did not induce radical production, see Fig. 7D), was inhibited only by inhibition of the MAPK-p38 pathway (Fig. 8B). This suggests that MAPK-p38 activation is involved in regulating both TLR2 gene expression and radical production.

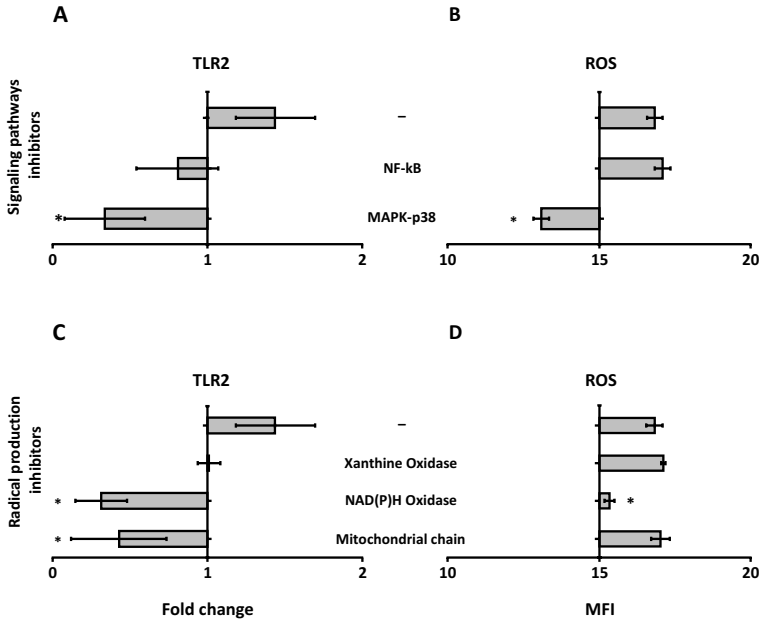

Figure 8. Effect of MAPK-p38 activation and ROS production on TLR2 gene expression.
Macrophages were pre-incubated for 30 min with inhibitors of NF-κB (PDTC, 5 μM), MAPK-p38 (SB203530, 25 μM), xanthine oxidase (allopurinol, 200 μM), NADPH oxidase (DPI, 20 μM) or mitochondrial electron transfer chain subunit I (rotenone, 10 μM). A. and C. Macrophages were further stimulated for 6h with PGN (50μg/ml). TLR2 gene expression was measured by means of real time quantitative PCR. 40S ribosomal protein gene was used as house keeping gene and results are expressed as mRNA fold change in stimulated cells relative to untreated cells (fold change=1). Averages ± SD of n=4 fish are given. Symbol '*' represents a significant ($P \leq 0.05$) difference compared to PGN-stimulated cells. B. and D. Macrophages were further incubated for 1h with DHR (0.25 μg/ml) and PMA (0.05 μg/ml) followed by PGN (50 μg/ml). Radical production was measured by means of DHR fluorescence intensity. Results are expressed as MFI relative to PMA-stimulated cells (MFI=15). Averages ± SD of n=4 fish are given. Symbol '*' represents a significant ($P \leq 0.05$) difference compared to PGN-stimulated cells.

Enzymatic pathways associated with radical production were studied by pre-incubation of carp macrophages with inhibitors of the mitochondrial electron transfer chain subunit I (rotenone), NAD(P)H oxidase (DPI) and xanthine oxidase (allopurinol). TLR2 gene expression, induced by PGN or LTA (not shown), could be inhibited by the presence of any of the three inhibitors, although to a lesser extent by the inhibitor of xanthine oxidase (Fig. 8C). Radical production, induced by PGN, was significantly inhibited only in the presence of the NAD(P)H oxidase inhibitor (Fig. 8D). This indicates that oxygen radicals, including the NAD(P)H oxidase-derived intracellular ROS (e.g.: H_2O_2), are involved in the regulation of TLR2 gene expression in carp macrophages.

H_2O_2 is an essential signaling molecule for TLR2 gene expression and MAPK-p38 activation

Reactive oxygen species, such as H_2O_2, are able to diffuse across membranes and therefore can act as signaling molecules (56). TLR2 gene expression in carp macrophages was significantly modulated by H_2O_2 in a concentration-dependent manner (Fig. 9A). Consistent with these findings, PGN-induced TLR2 gene expression in carp macrophages could be inhibited by a H_2O_2 scavenger (catalase) (Fig. 9B). To investigate if H_2O_2 influences TLR2 gene expression *via* MAPK-p38 activation, carp macrophages were stimulated with increasing concentrations of H_2O_2. Indeed, MAPK-p38 activation was considerably up-regulated by H_2O_2, in a concentration-dependent manner, within 5 min after addition of H_2O_2 (Fig. 9C). MAPK-p38 activation was significantly up-regulated by H_2O_2 at concentrations greater than 1 mM H_2O_2. The concentration of > 1mM H_2O_2 required to activate MAPK-p38 (57) implies that TLR2 gene expression is highly sensitive to modulation by H_2O_2 (0.1 mM, Fig. 9A). Our data indicate that H_2O_2 is able to induce TLR2 gene expression and activate the MAPK-p38 signaling cascade in carp macrophages. Futhermore, catalase (H_2O_2 scavenger) but not SOD (dismutates $O_2^{\cdot-}$ into H_2O_2) inhibited PGN-induced MAPK-p38 phosphorylation (data not shown). These observations suggest that NAD(P)H oxidase derived radicals such as H_2O_2, in particular, are essential for a complete MAPK-p38 phosphorylation and maximal expression of the TLR2 gene.

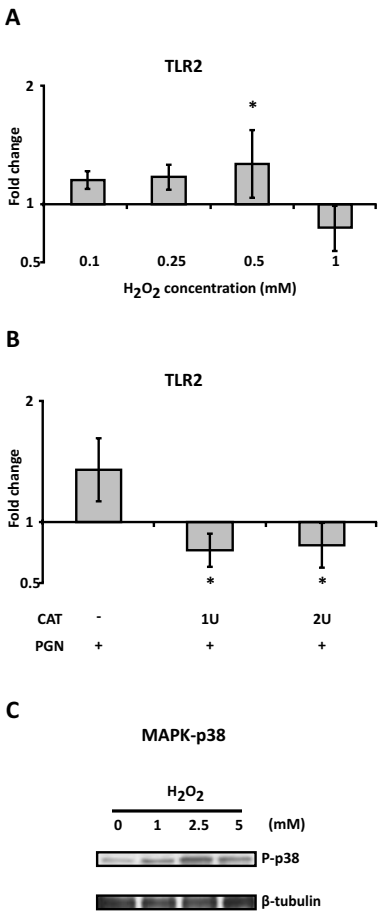

Figure 9. TLR2 gene expression and MAPK-p38 activation in carp macrophages under influence of hydrogen peroxide. A. TLR2 gene expression in carp macrophages after stimulation for 6h with H_2O_2 (0.1, 0.25, 0.5 and 1 mM). Averages and SD of n=4 fish are given. mRNA levels of TLR2 are relative to the house keeping 40S ribosomal protein gene level and expressed as fold change in stimulated cells relative to unstimulated cells (fold change=1). Symbol '*' represents a significant ($P \leq 0.05$) difference compared to unstimulated cells (control group). B. TLR2 gene expression in carp macrophages after pre-incubation with catalase (1 or 2U/ml) for 30 min followed by stimulation with PGN (50 μg/ml). Averages ± SD of n=4 fish are given. Symbol '*' represents a significant ($P \leq 0.05$) difference compared to PGN-stimulated cells. C. MAPK-p38 activation in carp macrophages incubated for 5 min with increasing concentrations of H_2O_2 (1, 2.5, 5 mM). MAPK-p38 phosphorylation was analyzed by immunoblotting for phospho-p38 (P-p38), while equal loading was confirmed by immunoblotting for β-tubulin.

DISCUSSION

Previous studies have identified TLR2 orthologues in teleost fish based on sequence homology, but the role of the TLR2 receptor in the recognition of ligands from Gram-positive bacteria has not been studied. Transfection of human cells (HEK 293) with the carp TLR2 receptor showed activation of MAPK-p38 by LTA and PGN from *Staphylococcus aureus*, which are prototypical TLR2 ligands from Gram-positive bacteria. The synthethic triacylated lipopeptide Pam_3CSK_4 but not the diacylated lipopeptide MALP-2 also activated TLR2 transfected human cells. Overexpression of TLR2 in carp macrophages confirmed the response to LTA and PGN, low-responsiveness to Pam_3CSK_4 and non-responsiveness to MALP-2. Activation of carp macrophages by LTA and PGN from *S. aureus* resulted in increased TLR2 gene expression and enhanced TLR2 mRNA stability. ROS production and MAPK-p38 activation cooperatively determined the level of TLR2 gene expression,

indicating that the H_2O_2-MAPK-p38-dependent axis is crucial for regulation of TLR2 gene expression in fish cells.

Similar to mammalian vertebrate TLR2, carp TLR2 is primarily expressed in myeloid cell types as a type I transmembrane protein composed of an extracellular, a transmembrane and an intracellular C-terminal domain. The extracellular domains of mammalian vertebrate TLR2 recognize LTA (6, 7), PGN (58) (59) (60) and lipopeptides (5). Mammalian vertebrate TLR2 contains 20 leucine rich repeats (39) whereas the extracellular domain of carp TLR2 is composed of 21 LRR motifs, one of which has a 'bacterial' motif and may not be important for ligand recognition. Although, in general, the extracellular LRR domain appears to have evolved more rapidly than the intracellular Toll/IL-1 receptor (TIR) domain (36), comparative sequence analysis revealed a conservation of the position of the critical PGN recognition leucine residues (50, 51, 61) in the carp TLR2 extracellular domain. Transfection of human (HEK 293) cells with carp TLR2 confirmed the ability of prototypical TLR2 ligands (LTA, PGN and Pam_3CSK_4) to trigger MAPK-p38 activation. In contrast, carp TLR2 did not recognize the TLR2 ligand MALP-2 in transfected HEK 293 cells.

TLR activation in humans is often measured via quantification of NF-κB activation or downstream expression of cytokines such as IL-1β and TNFα. We characterized activation of fish macrophages by determining the expression profile of several cytokine genes, ROS and NO production and MAPK-p38 phosphorylation (62). We identified similarities but also clear differences between the mammalian vertebrate and carp TLR2-mediated response. We observed in fish macrophages i) low responsiveness to Pam_3CSK_4, ii) non-responsiveness to MALP-2, iii) delayed gene expression kinetics and distinct cellular responses (radical production and MAPK pathway activation) to LTA as compared to PGN, iv) a requirement for at least 5-times higher doses of LTA and PGN than in humans.

i) The low responsiveness of fish cells to Pam_3CSK_4 is in contrast with the observation that carp TLR2-transfected HEK 293 cells clearly do bind Pam_3CSK_4. In mammalian vertebrates, Pam_3CSK_4 is recognized by a TLR2-TLR1 heterodimer (54). Apparently, overexpression of TLR2 homodimers in HEK 293 cells overcomes the requirement for TLR2-TLR1 heterodimerization and is sufficient for the recognition of Pam_3CSK_4. Overexpression of TLR2 homodimers in carp macrophages indeed improved recognition of Pam_3CSK_4 as shown by increased iNOS gene expression. The low responsiveness to Pam_3CSK_4 observed in carp is in line with previous reports in gilthead seabream (63) and rainbouw trout (35). In both studies a minor modulation of cytokine gene expression with Pam_3CSK_4 was observed, however, no additional prototypical TLR2 ligands were used.

ii) The unresponsiveness of fish cells to MALP-2, recognized by TLR2-TLR6 heterodimers in mammalian vertebrates (12), is consistent with the unresponsiveness of TLR2-transfected HEK 293 cells to MALP-2. Apparently, overexpression of TLR2 homodimers in HEK 293 cells does not overcome the requirement for TLR2-TLR6 heterodimerization. In mammalian vertebrates it is clear that distinct TLR2-containing receptor complexes allow for the accommodation of structurally diverse TLR2 ligands (62) and the ability of TLR2

to detect a relatively wide array of PAMPs has been attributed to a functional interaction with a number of other receptors (64, 65), including TLR1 and TLR6. TLR1, 6 and 10 are thought to have diverged from a common ancestral gene (66). The presence of a putative TLR1 homologue in fish (17, 67) is presently under investigation, which would allow for future studies on TLR2-TLR1 heterodimerization and detailed studies into recognition of triacylated lipopeptides. TLR6 does not seem to have an orthologue in teleost fish (17, 21). The apparent absence of a functional TLR6 homologue in carp could contribute to the unresponsiveness to MALP-2 observed in carp macrophages.

iii) LTA consistently stimulated downstream cytokine gene expression with a delayed kinetic profile when compared to PGN, an observation also made for mammalian vertebrates (62). PGN always more clearly than LTA induced NO, ROS production and MAPK-p38 phosphorylation. Differences in kinetics were previously suggested to be the result of distinct TLR2 co-receptors usage and signaling after cellular trafficking of these complexes (62). Although yet to be characterized, the distinct cellular responses could possibly be also attributed to the use of distinct TLR2-containing receptor complexes on fish macrophages. Different recruitment and/or kinetics of TLR(2)-specific adaptor proteins (68) upon activation of TLR2-containing complex may lead to ligand-specific responses and ultimately have profound effects on cellular response to LTA and PGN.

iv) Fish cells did respond to both LTA and PGN, albeit at high concentrations (35, 63, 69), which fact could not simply be ascribed to the ligand preparations because we used highly purified TLR2 ligands throughout our study. LTA from *S. aureus* was obtained by buthanol extraction which preserves its molecular structure (70, 71). PGN was a soluble polymeric high molecular-weight preparation purified from supernatants of *S. aureus* grown in the presence of penicillin, devoid of teichoic acids or other proteins (72). As stated above, the ability of TLR2 to detect a relatively wide array of PAMPs has been attributed to a functional interaction with a number of other receptors (64, 65). These not only include TLR1 and TLR6, but also the lipid scavenger receptor CD36 and the CD14 protein. A CD36 gene sequence is present in zebrafish but no functional studies have been reported on the formation of a functional receptor complex of fish TLR2 with the CD36 lipid scavanger receptor. LTA and PGN also can interact with CD14 (73) (74), (75-77). A recent report (78), in fact, suggests that the main function of CD36 is to bind and transfer diacylglycerol ligands (e.g.: lipomannan, LTA) onto TLR2/TLR6, in a CD14-dependent manner. So far, CD14 has not been detected in any fish genome or EST database, suggesting the absence of this co-receptor in fish. If true, the absence of CD14 could not only contribute to the well-known hyporesponsiveness of fish cells to Gram-negative (26, 79) bacteria but also to the hyporesponsiveness of fish cells to ligands from Gram-positive bacteria reported here.

Of course, carp macrophages undoubtly express innate receptors additional to TLR2 and it is impossible to unambiguously ascribe activation of carp macrophages by TLR2 ligands to the activation of TLR2 receptors only. In mammalian vertebrates, several families of PRRs have been shown to detect PGN from Gram-positive bacteria including TLR2 (60), nucleotide-binding oligomerization domain 2 (NOD2) and peptidoglycan

recognition proteins (PGRPs) (80). In zebrafish it was shown that suppression of PGRP6 decreased significantly the expression of TLR2 mRNA suggesting that TLR2 and PGRP may cooperatively recognize PGN (81). Without access to knock-out phenotypes it remains difficult to exclude the involvement of other receptors on carp macrophages, such as NOD2. However, stimulation of carp macrophages with muramyl dipeptide (MDP); the minimum PGN fragment recognized by NOD2 (82), did not induce ROS and NO to the same extent as PGN (unpublished observation). This suggests that NOD2 did not play the major role in the stimulation by PGN we observed in carp macrophages. Furthermore, overexpression of TLR2 in carp macrophages led to a more pronounced induction of downstream cytokine gene expression in response to LTA , PGN and, to a minor extent, Pam_3CSK_4. These results corroborate the ability of carp TLR2 to bind LTA and PGN from *S. aureus* and to trigger TLR2-dependent downstream activation pathways in response to these ligands in carp macrophages. In conclusion, we provide evidence that fish macrophages can be activated by ligands from Gram-positive bacteria that are prototypical activators of mammalian vertebrate TLR2, but require relatively high concentrations.

We investigated if TLR2 ligands could modulate the expression of the carp TLR2 gene itself. Clearly, carp TLR2 gene expression was regulated in a consistent manner throughout our studies, although to a maximum of 2-fold upregulation. In mammalian vertebrates, expression patterns of the TLR2 gene itself are divergent. In human monocytes, TLR2 mRNA is upregulated after adherence to tissue culture plates but cannot be further induced. (83). In murine monocytes, TLR2 mRNA is low or undetectable in vitro but can be strongly induced (84). In cell lines, two- to three-fold increase of TLR2 gene expression has been observed in murine (RAW 264.7) and human (HL-60) macrophages (85). In our study, GPI anchors from protozoan parasites of carp showed a dose-dependent up-regulation of TLR2 gene expression. It is known that the carp protozoan *T. borreli* parasite induces radical production in carp macrophages (86). We are currently defining the involvement of TLR2 in the recognition of GPI anchors from protozoan parasites. Further studies will elucidate the contribution of TLR2 to the immune response induced by protozoan parasites in carp. To further investigate the regulation of TLR2 gene expression, we studied if TLR2 ligands could induce post-transcriptional stabilization of carp TLR2 mRNA. Indeed, LTA and PGN specifically increased TLR2 mRNA stability which could contribute to the consistent 2-fold inducibility of TLR2 gene expression. Changes in mRNA stability for TLR2 in mammalian vertebrates are yet to be reported. Stimulation of carp macrophages with PGN clearly induced MAPK-p38 phosphorylation and increased NO and ROS production. MAPK-p38 activation was dependent on NAD(P)H oxidase derived radicals, in particular H_2O_2, suggesting that the MAPK-p38 activation pathway in fish is redox-sensitive. Complementary, PGN-induced radicals were NAD(P)H oxidase as occurs in humans (87). Moreover, PGN-induced radicals could be further increased by the presence of superoxide dismutase, suggesting that H_2O_2 production occurs following TLR2 engagement. We found that PGN-induced radicals derived from NAD(P)H oxidase were necessary for maximal expression of carp TLR2 gene expression. Furthermore, catalase significantly inhibited

TLR2 gene expression while H_2O_2 significantly induced TLR2 gene expression. In our study, MAPK-p38 was required for an effective PGN-induced TLR2 expression and post-transcriptional stability, suggesting a clear role for MAPK-p38 in the regulation of TLR2 gene expression in carp. Collectively, these results suggest that H_2O_2 radicals *via* MAPK-p38 activation play an indispensable role in the regulation of TLR2 gene expression itself in carp macrophages. In mice, ROS-dependent activation of the TRAF6-ASK1-p38 pathway is selectively required for TLR4 innate immunity (88). In humans, ASK1-MAPK-p38-p47 phox activation is essential for inflammatory responses during tuberculosis *via* TLR2-ROS signaling (89). These observations suggest an important role for ROS as second messengers in TLR-mediated signaling pathways. We show an important role for ROS, in particular H_2O_2, on TLR2 gene expression in carp macrophages. We demonstrated for the first time, in carp, that bi-directional communication between ROS and activated MAPK-p38, besides shaping the TLR-mediated response, has a direct effect on the level of TLR2 gene expression.

ACKNOWLEDGMENTS

We thank the central fish facilities, "De Haar-Vissen," for providing and taking care of the carp. Prof. N. Okamoto (Department of Aquatic Biosciences, Tokyo University of Fisheries, Tokyo, Japan) is acknowledged for providing the mouse mAb TCL-BE8. Magdalena Chadzinska, Tosca Ploegaert, Natalia Ivonne Vera Jimenéz, Jerry Wells, Irene Konings, Henk Schipper, Jochem van der Veen, and Maria Forlenza are gratefully acknowledged for their practical assistance or fruitful discussions.

FUNDING

This work was supported by the Portuguese foundation Fundação para a Ciência e Tecnologia under Grant agreement number SFRH/21522/2005 and by the European Community's Seventh Framework Programme ([FP7/2007-2013] under Grant agreement number PITN-GA-2008-214505

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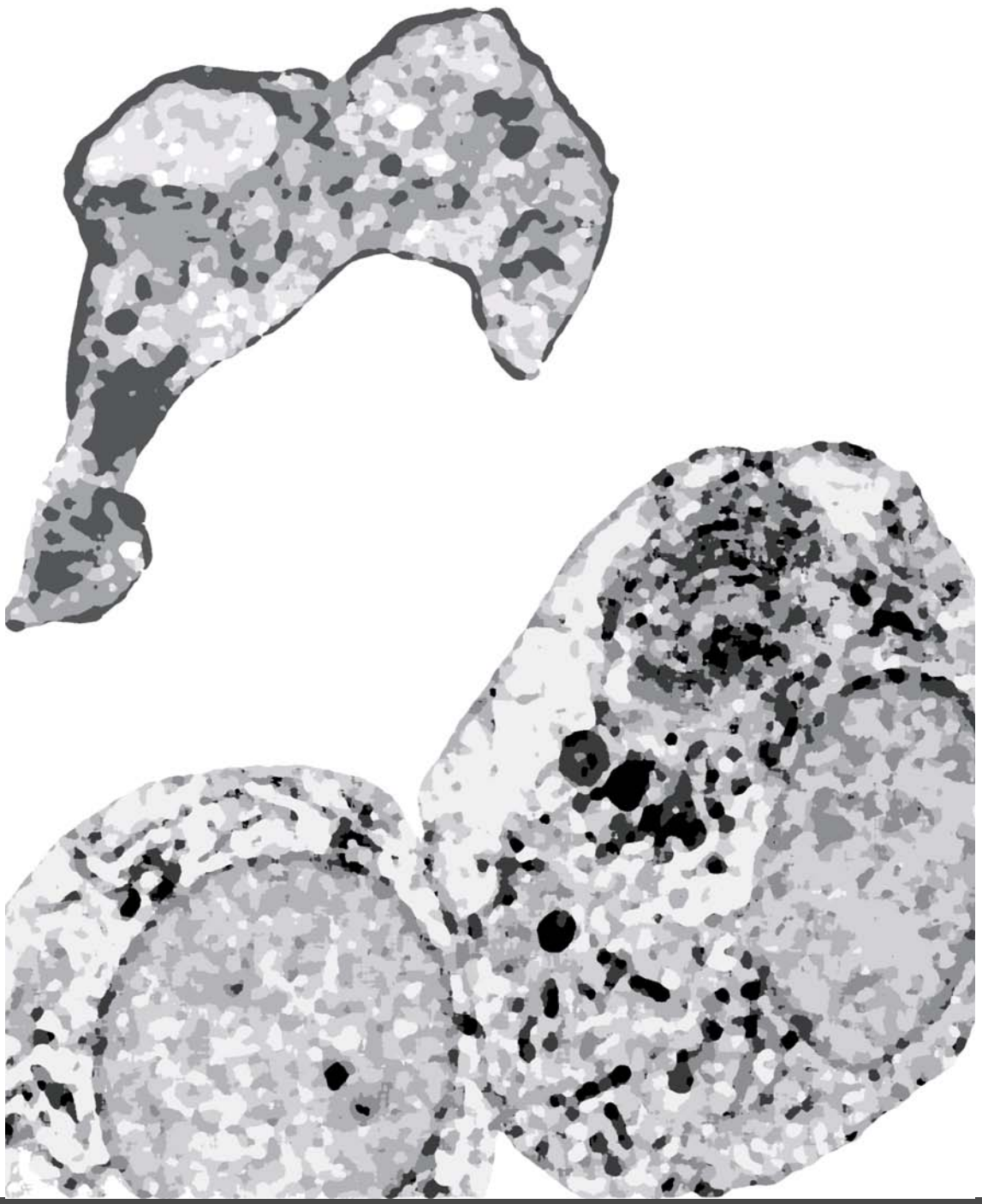
" Stones in the road?
I save every single one, one day I'll build a castle. "

Fernando Pessoa



Chapter 5

Trypanosomiasis-induced Th17-like immune responses in carp

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PLoS One, In press

ABSTRACT

In mammalian vertebrates, the cytokine interleukin (IL)-12 consists of a heterodimer between p35 and p40 subunits whereas interleukin-23 is formed by a heterodimer between p19 and p40 subunits. During an immune response, the balance between IL-12 and IL-23 can depend on the nature of the pathogen associated molecular pattern (PAMP) recognized by, for example TLR2, leading to a preferential production of IL-23. IL-23 production promotes a Th17-mediated immune response characterized by the production of IL-17A/F and several chemokines, important for neutrophil recruitment and activation. For the cold blooded vertebrate common carp, only the IL-12 subunits have been described so far. Common carp is the natural host of two protozoan parasites: *Trypanoplasma borreli* and *Trypanosoma carassii*. We found that these parasites negatively affect p35 and p40a gene expression in carp. Transfection studies of HEK293 and carp macrophages show that *T. carassii*-derived PAMPs are agonists of carp TLR2, promoting p19 and p40c gene expression. The two protozoan parasites induce different immune responses as assessed by gene expression and histological studies. During *T. carassii* infections, in particular, we observed a propensity to induce p19 and p40c gene expression, suggestive of the formation of IL-23. Infections with *T. borreli* and *T. carassii* lead to an increase of IFN-γ2 gene expression whereas IL-17A/F2 gene expression was only observed during *T. carasssii* infections. The moderate increase in the number of splenic macrophages during *T. borreli* infection contrasts the marked increase in the number of splenic neutrophilic granulocytes during *T. carassii* infection, along with an increased gene expression of metalloproteinase-9 and chemokines. This is the first study that provides evidence for a Th17-like immune response in fish in response to infection with a protozoan parasite.

INTRODUCTION

In mammalian vertebrates, the three members of the IL-12 cytokine family comprise the heterodimeric IL-12, IL-23 and IL-27; all molecules belonging to the type I cytokine superfamily. IL-12 consists of two disulphide-linked proteins: the p35 and the p40 subunit, together forming IL-12p70 (1). The p40 subunit also participates in the formation of IL-23, formed in combination with a subunit related to p35, named p19 (2). The last member of the family, IL-27, consists of a heterodimer between two other subunits, although both with homology to the p35 and p40 subunits, respectively, named p28 and EBI3 (3). Because IL-12 and IL-23 share the IL-12Rβ1 receptor subunit, the ability of cells to respond to either IL-12 or IL-23 is determined by the expression of the other chain; IL-12Rβ2 or IL-23R, respectively (4), (5). IL-27 signals also through a heterodimeric receptor composed of IL-27Rα and gp130, the latter is shared with the IL-6 receptor (3). Thus, despite their

structural similarities, based on recognition by distinct heterodimeric receptors on the cell membrane, IL-12, IL-23 and IL-27 have distinct functions.

For the cold blooded vertebrate common carp (*Cyprinus carpio* L.), not all IL-12 cytokine family members have been described. Sequences for both p35 and p40 were found, suggestive of the formation of an IL-12 molecule in carp. The subunit p35 so far was reported as a single gene, whereas three distinct p40 genes (p40a, p40b and p40c) were described (6), (7). Major differences between the three p40 isoforms in constitituve gene expression and *in vitro* inducibility, indicate differences in function. Of the three carp p40 isoforms, p40a seems most similar to mammalian p40 proteins with respect to amino acid sequence identity and conservation of critical residues for heterodimerisation with p35 (6). Upregulation of gene expression of p35 and p40b, in particular, is suggestive of the formation of an IL-12-like molecule as a signal-3 cytokine during viral infection (8). With regard to the third isoform, p40c, phylogenetic analysis indicates that p40a and p40b share a common ancestor following their divergence from p40c. More importantly, the cysteine residue in the human p40 molecule that forms a stabilizing interchain disulphide bridge with p35 is not present in carp p40c (6). Only very recently the subunit p19 was described as a molecule likely involved in the IL-23/Th17-driven immune response in zebrafish (*Danio rerio*) (9). Carp and zebrafish are closely-related species that belong to the the same Family (Cyprinidae) and have similar immunological responses. In the present manuscript, we studied gene expression of different IL-12 cytokine family members, including p19, p35 and p40a-c, in response to protozoan infection of carp.

In mammalian vertebrates, IL-12 constitutes an important factor in the differentiation and expansion of Th1 cells, which produce IFN-γ that activates myeloid cells to secrete TNFα, both essential for defense against intracellular pathogens. IL-23 is required for the expansion of Th17 cells, which produce IFN-γ but also IL-17A and IL-17F, essential for the defense against extracellular bacteria and fungi (10), (11). Thus, Th17-mediated immune responses are IL-23 driven and characterized by the production of IL-17A/F and several chemokines, all important for neutrophil recruitment and activation (12). During infections, the balance between IFN-γ and IL-17A/F cytokines represents the balance between a Th1 and a Th17 response (13). In carp, IFN-γ exists as two distinct genes with IFN-γ2, in particular, being associated with characteristic T-lymphocyte function and classical phagocyte activation. The exact function of IFN-γrel (previously referred to as IFN-γ1) is presently unknown. Zebrafish IL-17 A/F exists as three distinct genes with IL-17A/F2, in particular, being the only IL-17A/F gene expressed in both systemic (kidney) and mucosal (intestine and gills) immune tissue (14). In the present manuscript, we describe the possibility that formation of carp IL-23 can lead to a Th17-like immune response to a protozoan parasite of common carp.

Professional antigen presenting cells are the prime source of all three IL-12 cytokine family members, at least in mammalian vertebrates. The balance between the three IL-12 cytokine family members during the course of an immune response is dependent on the nature of the pathogen associated molecular pattern (PAMP) recognized (15),

(16), (17). For example in mammalian vertebrates, TLR2 activation by bacterial-derived peptidoglycan (PGN) induces high levels of IL-23 but not IL-12 (15). We recently identified carp TLR2 and described its involvement in the recognition of bacterial PAMPs such as PGN but also of GPI-anchors from protozoan parasites (18). Carp is the natural host of two extracellular protozoan blood parasites: *Trypanoplasma borreli* and *Trypanosoma carassii.* Both *T. borreli* are *T. carassii* are believed to live extracellular in the blood and tissue fluids of their fish hosts (19) (20). The immune response of carp against these two parasites is fundamentally different. *T. borreli* infections are associated with a type 1-like immune response, characterized by a classical IFN-γ2-mediated activation of macrophages leading to a high production of TNFα and NO within 3 weeks (21), (22), (23). In contrast, *T. carassii* infections of carp do not lead to an excessive NO response, extend over a 2-3 weeks longer infection period and are associated with an alternative activation of macrophages (22), (24), (25). In the present manuscript, we describe that *T. carassii*, in particular, induces a Th17-like immune response in carp.

MATERIALS AND METHODS

Ethic statement

All animals were handled in strict accordance with good animal practice as defined by the relevant national and/or local animal welfare bodies, and all animal work was approved by the animal experimental committee of Wageningen University, Wageningen, The Netherlands. (license numbers: 2004079/2004137/2008054)

Animals

European common carp (*Cyprinus carpio carpio* L.) were reared in the central fish facility of Wageningen University, The Netherlands at 23 °C in recirculating UV-treated tap water and fed pelleted dry food (Skretting, Nutreco) daily. R3xR8 carp are the hybrid offspring of a cross between fish of Hungarian origin (R8 strain) and of Polish origin (R3 strain) (26). Carp were between 9 and 11 months old at the start of the experiments.

Protozoan parasites and GPI-anchors

Trypanoplasma borreli was cloned and characterized by Steinhagen *et al*. (19). *Trypanosoma carassii* was cloned and characterized by Overath *et al.* (27). Both parasites were maintained by syringe passage through carp. Parasitaemia was monitored in 10 x diluted blood in cRPMI [RPMI 1640 (Invitrogen, CA, USA) adjusted to carp osmolarity 280 $mOsmkg^{-1}$ containing 50 U/ml of heparin (Leo Pharma BV, Weesp, The Netherlands)] using a Bürker counting chamber. The minimum detection limit by this method was 10^5 parasites/ml of blood. For parasite isolation, blood was collected from 3-weeks-infected fish and purified on a 1x12cm ion-exchange chromatography using DEAE cellulose (DE-52; Whatman international) (27). After purification, parasites

were resuspended in HML medium (28) supplemented with 5% pooled carp serum, L-glutamine (2 mM, Cambrex, Verviers, Belgium), penicillin G (100 U/ml, Sigma-Aldrich, Zwijndrecht, The Netherlands), and streptomycin sulfate (50 mg/l, Sigma-Aldrich).

GPI-anchors from the parasites were obtained as described previously (18). In short, equal numbers of *T. borreli* or *T. carassii* parasites were incubated for 30 min at 30 ºC in 30 % Tris-buffer (10 mM Tris-HCl, 144 mM NaCl, 0.05% BSA, pH=7.4) or in 1 unit of phosphatidylinositol-specific phospholipase C [PI-PLC from *Bacillus cereus* (Sigma-Aldrich)] in 30% Tris-buffer. Samples were centrifuged at 800 *g* for 10 min and supernatants were collected and filter-sterilized (0.22 µm Millex-GV, Millipore, Ireland). Pellets from PI-PLC-treated parasites were collected and resuspended in incomplete NMGFL-15 medium. Carp macrophages were stimulated with 25 µL (1:4) of each fraction. HEK 293 cells were stimulated with 50 µL (1:5) of each fraction.

Experimental setup of infection of carp with protozoan parasites

In vivo infections with *T. borreli* or *T. carassii* were performed as described previously (8) and (24), respectively). Before the start of each infection experiment fish were moved to a quarantine facility and acclimatized to 20°C over a period of at least 2 weeks. Fish were euthanized with 0.3 g/l tricaine methane sulfonate (TMS, Crescent Research Chemicals, Phoenix, AZ, USA) buffered with 0.6 g/l $NaHCO_3$ (Merck, Darmstadt, F.R. Germany) prior to sampling blood, head kidney or spleen.

For gene expression studies in parasite-infected fish, carp were i.p. injected with 10^4 *T. borreli* parasites per fish in 100 µL or with PBS (unchallenged control group) and head kidneys of n=5 infected and n=3 non-infected fish sampled per time point, over a period of 6 weeks post-infection. In a separate experiment, carp were i.p. injected with 10^4 *T. carassii* parasites per fish in 100 µL or with PBS and head kidneys of n=4 (non-) infected fish sampled per time point, over a period of 10 weeks.

Recall experiments with parasite-infected carp

For *ex vivo* recall experiments with phagocytes from parasite-infected carp, carp were i.p. injected with 10^4 parasites (*T. borreli* or *T. carassii*) per fish in 100 µL, or with PBS, and head kidneys of n=3 infected and n=1 non-infected fish sampled per time point, over a period of 5 weeks. Blood was collected by puncture of the caudal blood vessel by a syringe containing heparinised RPMI 1640; a small aliquot of blood was used to determine parasitaemia using a Bürker counting chamber and the remainder was used to isolate peripheral blood leukocytes (PBL). At the same time, spleens and head kidneys were aseptically removed. Spleens were snap frozen in liquid nitrogen and stored at -80°C until use. Head kidneys were used to isolate head kidney leukocytes (HKL).

For PBL isolation, blood was centrifuged first for 15 min at 800 *g* to remove the red blood cells. The buffy coat containing the leukocytes was collected and layered on 3 ml Ficoll-Paque™ Plus (Amersham Biosciences, Uppsala, Sweden). Following subsequent centrifugation at 800 *g* for 25 min with the brake disengaged, leukocyte layer at the

interface was collected and washed three times with cRPMI. Cell pellets were collected, directly lysed and stored at -80°C prior to RNA isolation.

HKL isolation was performed as previously described (25). Briefly, head kidneys were gently passed through a 100 µm sterile nylon mesh (BD Biosciences, Breda, The Netherlands) and rinsed with homogenization buffer [incomplete NMGFL-15 medium containing 50 U/ml penicillin G, 50 µg/ml streptomycin sulphate, and 20 U/ml heparin. Cell suspensions were layered on 51% (1.07 $g.cm^{-3}$) Percoll (Amersham Biosciences, Uppsala, Sweden) and centrifuged at 450 *g* for 25 min at 4°C with the brake disengaged. HKL at the interphase were collected and washed twice in incomplete NMGFL-15. *Ex vivo* recall stimulations were performed with HKL ($0.5x10^6$ per well) seeded in 100 µL rich-NMGFL-15 medium [incomplete NMGFL-15 medium supplemented with 2.5% heat-inactivated pooled carp serum and 5% fetal bovine serum (FBS, Invitrogen)] in a 96-well culture plate. HKL were stimulated with peptidoglycan (50 µg/mL, soluble secreted PGN from *S. aureus*, Invivogen, Cayla SAS, France), live *T. borreli* or *T. carassii* ($0.25x10^6$ parasites per well) for 6 h at 27°C prior to RNA isolation, or left untreated.

Macrophage cell cultures

Head kidney-derived macrophages, considered the fish equivalent of bone marrow-derived macrophages, were prepared as previously described (25), (29). Briefly, carp head kidneys were gently passed through a 100 µm sterile nylon mesh, rinsed with homogenization buffer. Cell suspensions were layered on 51% (1.07 $g.cm^{-3}$) Percoll and centrifuged at 450 *g* for 25 min at 4°C with the brake disengaged. HKL at the interphase were removed and washed twice in incomplete NMGFL-15 medium. Macrophage cell cultures were initiated by seeding 1.75 x 10^7 head kidney leukocytes in a 75 cm^2 culture flask containing 20 ml of complete NMGFL-15 medium [incomplete NMGFL-15 medium supplemented with 5% heat-inactivated pooled carp serum and 10% FBS]. Head kidney-derived macrophages, named macrophages throughout the manuscript, were harvested after 6 days of incubation at 27 °C by placing the flasks on ice for 10 min prior to gentle scraping.

Purification of neutrophilic granulocytes

Total leukocytes from spleen were isolated essentially as described for head kidney total leukocytes (30). Cell suspensions were layered on a discontinous Percoll gradient (1.020 and 1.083 g cm^{-3}) and centrifuged 30 min at 800 *g* with the brake disengaged. Cells at the interface 1.020 and 1.083 g cm^{-3} were collected and washed twice with cRPMI. The monoclonal antibody TCL-BE8 (1:50) (31) was used to purify neutrophilic granulocytes by magnetic sorting (18). After incubation for 30 min with TCL-BE8 on ice, the leukocyte suspension was washed twice with cRPMI and incubated with phycoerythrin (PE)-conjugated goat anti-mouse (1:50; DAKO, Glostrup, Denmark) 30 min on ice. After washing twice, the total cell number was determined with a Bürker counting chamber, and 10 µl of magnetic beads (anti-PE Microbeads, Miltenyi Biotec, GmbH, Germany) was added per 10^8 cells. After incubation for 15 min at 4 °C, cells were washed twice and resuspended in

cRPMI. The magnetic separation was performed on LS-MidiMACS Columns (Mitenyi Biotec) according to the manufacturer's instructions. For RNA isolation, TCL-BE8$^+$ cells were resuspended in 1 ml of cRPMI and directly lysed. The purity of the TCL-BE8$^+$ neutrophilic granulocyte-enriched fraction was > 90% as confirmed by flow cytometric analysis using a FACScan® flow cytometer (Becton Dickinson, Mountain View, CA, USA).

Immunohistochemistry

Cryosections (7 µM) of spleen tissue were mounted on poly-*L*-lysine-coated glass slides (BDH Laboratory Supplies, Poole, UK), air-dried for 60 min and incubated in a 0.3 % H_2O_2 solution in methanol for 20 min to inactivate endogenous peroxidase. Following steps were performed at RT unless stated otherwise. Cryosections were washed for 5 min with PBS, then short with distilled water and incubated in proteinase-K solution (50 µg/ml in distilled water) for 10 min at 37°C. Samples were fixed in 4% paraformaldehyde in PBS for 10 min at 4°C followed by washing in 0.1 % Triton PBS (PBS-T) for 10 min at 4°C and subsequently in PBS-T for 7 min at RT. Cryosections were first blocked in 5 % normal goat serum for 30 min and then incubated with primary antibody in PBS for 1 h. Mouse monoclonal antibodies were used to detect neutrophilic granulocytes (TCL-BE8, 1:50) (31) and macrophages (WCL-15, 1:50) (32), (33). After washing twice for 10 min in PBS-T, sections were incubated with goat anti-mouse horseradish peroxidase-conjugated (GAM-HRP, 1:200, Dako, Glostrup, Denmark) in PBS for 1 h. After washing twice in PBS-T, sections were incubated for 10 min in 0.05 M sodium acetate buffer, pH 5 and following addition of 0.4 mg/ml 3-amino-9-ethyl-carbazole (AEC; Sigma-Aldrich) in sodium acetate buffer containing 0.03 % H_2O_2 and incubated for 25 min. Finally, cryosections were rinsed four times in distilled water and embedded in Kaiser's glycerine gelatin (Merck, Darmstadt, Germany).

TLR2 WT-GFP and TLR2∆TIR-GFP expression plasmids

Amplification of carp TLR2WT (wild type; full-length sequence) and TLR2ΔTIR (sequence truncated at TIR domain) cDNA was performed as previously described (18). The vivid color™pcDNA™6.2/C-EmGFP-GW/TOPO® (Invitrogen, catalog no. K359-20) expression vector combined with TOPO®cloning was used to fluorescently label the construct by fusing TLR2 WT or TLR2ΔTIR to EmGFP at the C-terminal end. Isolation of highly pure plasmid DNA suitable for transfection was performed using S.N.A.P.™ Midi Prep Kit (Invitrogen, catalog no. K1910-01), according to the manufacturer´s protocol. Fluorescence-tagged protein was visualized using confocal microscopy.

Transient transfection of HEK 293 cells and carp macrophages

HEK 293 cells were cultured in DMEM supplemented with 10 % FBS, 50 U/ml penicillin G and 50 µg/ml streptomycin sulphate. Two days prior to transfection, HEK 293 cells were seeded into tissue culture flasks to reach 80-90 % confluence at the day of transfection. For transfection of HEK 293 cells, 2.5 µg of the carp TLR2 WT-GFP or TLR2ΔTIR-GFP constructs

was transfected into HEK 293 by nucleoporation using nucleofactor™ solution V and program A-23 (Lonza Cologne AG, Germany) according to the manufacturer´s instructions. Forty-eight hours after transfection, cells were trypsinized (0.5% trypsin, GIBCO) and plated overnight in a 24-well plate (18). The next day, cells were stimulated for 15 min with live protozoan parasites *(T. borreli* or *T. carassii*; 0.5 x 10^6 per well) or GPI-enriched fractions from 5x10^6 parasites *(T. borreli* or *T. carassii*, 50 µl per well), or left untreated as negative control. Cells were lysed for evaluation of phospho-p38 activity by Western blot.

For transfection of carp macrophages, 2.5 µg of the carp TLR2 WT-GFP or TLR2ΔTIR-GFP constructs was transfected by nucleoporation using nucleofactor™ Human Macrophage Solution and program Y-001 (Lonza Cologne AG, Germany) according to the manufacturer´s instructions and placed into a 48-well plate (18). After 24 h incubation, the medium was replaced and macrophages were stimulated for 6 h with live parasites *(T. borreli* or *T. carassii*; 0.5 x 10^6 per well) or GPI-enriched fractions from 5x10^6 parasites *(T. borreli* or *T. carassii*, 50 µl per well). Cells were lysed for RNA isolation.

RNA isolation and cDNA synthesis

RNA was isolated using the RNeasy Mini Kit (Qiagen, Leusden, The Netherlands) including the accompanying DNase I treatment on the columns, according to the manufacturers´ protocol. Final elution was performed with 30 µl nuclease-free water. RNA concentrations were measured by spectrophotometry (Nanodrop, Thermo scientific, Breda, The Netherlands) and 1 µl was analysed on a 1 % agarose gel to check the RNA integrity. RNA was stored at -80°C until further use. Prior to cDNA synthesis, a second DN*ase* treatment was performed using DN*ase* I, Amplification Grade (Invitrogen). Briefly, 1 µg of RNA from each sample was combined with 10X DN*ase* reaction buffer and 1 U DN*ase* I, mixed and incubated at RT for 15 min, followed by inactivation of DN*ase* I by adding 1 µl of 25 µM EDTA. Synthesis of cDNA was performed with Invitrogen's SuperScript™ III First Strand Synthesis Systems for RT-PCR Systems, according to the manufacturer's instructions. Briefly, DN*ase* I-treated RNA samples were mixed with 5x first strand buffer, 300 ng random primers, 10 µM dNTPs, 0.1 M DTT, 10 U RN*ase* inhibitor, and 200 U SuperScript III Reverse Transcriptase up to a final volume of 20 µl. The mixture was incubated at 37 °C for 60 min followed by an inactivation step at 70 °C for 15 min. A non-reverse transcriptase control was included for each sample. cDNA samples were further diluted 50 times in nuclease-free water before use as template in real-time PCR experiments.

Identification of carp p19 and IL-17A/F2 cDNA

Based on previously described sequences for zebrafish p19 (GenBank Accession number: ACC77208) and zebrafish IL-17A/F1-3 (GenBank Accession numbers:NP_001018623 [A/F1] NP_001018634 [A/F2] NP_001018626 [A/F3]), primers were designed to amplify the corresponding cDNA regions for p19 and IL-17A/F2 in common carp.

cDNA from head kidney and mid-kidney from *T. carassii* infected fish was used as template

for PCR to clone carp p19 and IL-17A/F2, respectively. A first PCR round was performed using the following primers to amplify p19: p19Fw-GCCTTCAAAGCAACAAAAAGACTT and p19Rv-GGAGTAGAGTCTTTCCACGCTGT. A first PCR round was performed using the following primers to amplify IL-17A/F2: IL-17A/F2: IL-17A/F2Fw-GTCTGCGTGGAACTGGATACCGAA and IL-17A/F2Rv-CAGCACCAGTATGTCCTGATAAATG. A second round using the same primer combination was performed to obtain a partial carp p19 (GenBank accession number: HM231139, 255 bp) and carp IL-17A/F2 cDNA (GenBank accession number: HM231140, 262 bp).

PCR reactions were performed in *Taq* buffer, using 1U *Taq* polymerase (Promega, Leiden, The Netherlands) supplemented with $MgCl_2$ (1.5 mM), dNTPs (200 µM) and primers (400 nM each) in a total volume of 50 µl. PCR and nested PCR were carried out under the following conditions: one cycle 4 min at 96 °C; followed by 35 cycles of 30 sec at 96 °C, 30 sec at 55 °C and 2 min at 72 °C; and final extension for 7 min at 72 °C, using a GeneAmp PCR system 9700 (PE Applied Biosystems, Foster City, CA). Products amplified by PCR, nested PCR or RACE-PCR were ligated and cloned in JM-109 cells using the pGEM-Teasy kit (Promega) according to the manufacturer´s protocol. From each product both strands of eight clones were sequenced, using the ABI prismBigDye Terminator Cycle Sequencing Ready Reaction kit and analysed using 3730 DNA analyser.

Real-time quantitative PCR (RT-qPCR)

Real time quantitative PCR (RT-qPCR) was performed in a 72-well Rotor-Gene™ 2000 (Corbett Research, Mortlake, Sydney, Australia) with the Brilliant® SYBR® Green QPCR (Stratagene, La Jolla, CA, USA) as detection chemistry. Primers used for RT-qPCR (Table I) were designed with Primer Express software. IL1-β and TNF-α primer sets were designed to amplify all known isoforms for each gene.

Master-mix for each PCR run was prepared as follows: 0.32 µl of water, 0.84 µl of each primer (5 µM), 7 µl Master SYBR Green I mix. To 5 µL of diluted cDNA, 9 µl of master mixed was added in a 0.1 ml tube. Following amplification program was used: one denaturation step of 15 min at 95 °C; followed by 40 cycles of RT-qPCR with three-step amplification (15 s at 95 °C for denaturation, 30 s at 60 °C for annealing and 30 s at 72 °C for elongation) and a final holding step of 1 min at 60 °C. A melting step was then performed with continuous fluorescence acquisition starting at 60 °C with a rate of 1 °C/5 s up to 99 °C to determine the amplification specificity. In all cases, the amplifications were specific and no amplification was observed in negative controls (non-template control and non-reverse transcriptase control). Fluorescence data from RT-qPCR experiments were analysed using Rotor-Gene version 6.0.21 software and exported to Microsoft Excel. The cycle threshold C_t for each sample and the reaction efficiencies (E) for each primer set were obtained upon Comparative Quantitation Analysis from the Rotor-Gene version 6.0.21 software. Briefly, the E for each primer set was recorded per sample and an average E (E_A) was then calculated for each primer set. The relative expression ratio (R) of a target gene was calculated based on the E_A and the C_t deviation of sample versus control, and expressed

in comparison to a reference gene (34), (35).

Table I: Primers used for real-time quantitative PCR analysis

Primer	Sequence (5'-3')	GenBank Accession No.
40S Fw	CCGTGGGTGACATCGTTACA	AB012087
40S Rv	TCAGGACATTGAACCTCACTGTCT	
TLR2 Fw	TCAACA+CTCTTAATGTGAGCCA[a]	FJ858800
TLR 2 Rv	TGTG+CTGGAAA+GGTTCAGAAA[a]	
IL-1β Fw	AAGGAGGCCAGTGGCTCTGT	AJ245635
IL-1β Rv	CCTGAAGAAGAGGAGGAGGCTGTCA	
TNF-α1,2 Fw	GCTGTCTGCTTCACGCTCAA	AJ311800-01
TNF-α1,2 Rv	CCTTGGAAGTGACATTTGCTTTT	
p19 Fw	CTCGCTCTGAAAAACTA+CACCAGG[a]	HM231139
p19 Rv	GGCACGCTCTCTC+CACTTACT[a]	
p35 Fw	TGCTTCTCTGTCTCTGTGATGGA	AJ580354
p35 Rv	CACAGCTGCAGTCGTTCTTGA	
p40a Fw	GAGCGCATCAACCTGACCAT	AJ621425
p40a Rv	AGGATCGTGGATATGTGACCTCTAC	
p40b Fw	TCTTGCACCGCAAGAAACTATG	AJ628699
p40b Rv	TGCAGTTGATGAGACTAGAGTTTCG	
p40c Fw	TGGTTGATAAGGTTCACCCTTCTC	AJ628700
p40c Rv	TATCTGTTCTACAGGTCAGGGTAACG	
CxCa Fw	CTGGGATTCCTGACCATTGGT	AJ421443
CxCa Rv	GTTGGCTCTCTGTTTCAATGCA	
CxCb Fw	GGGCAGGTGTTTTTGTGTTGA	AB082985
CxCb Rv	AAGAGCGACTTGCGGGTATG	
CXCL8_L2 Fw	TCACTTCACTGGTGTTGCTC	AB470924
CXCL8_L2 Rv	GGAATTGCTGGCTCTGAATG	
CxCR1 Fw	GCAAATTGGTTAGCCTGGTGA	AB010468
CxCR1 Rv	AGGCGACTCCACTGCACAA	
CxCR2 Fw	TATGTGCAAACTGATTTCAGGCTTAC	AB010713
CxCR2 Rv	GCACACACTATACCAACCAGATGG	
MHC-II DAB1-2 Fw	ACAGCTCCCGTGATTTCAGT	Z47731-32
MHC-II DAB1-2 Rv	CTCTGCGTTATATATACTCCAAGTGC	
MHC-II DAB3-4 Fw	GCGTTTCAGGCGGACTCTT	Z47733
MHC-II DAB3-4 Rv	ACACCATATCACTGTAATCACT	
MMP9 Fw	ATGGGAAAGATGGACTGCTG	AB057407
MMP9 Rv	TCAAACAGGAAGGGGAAGTG	
IFNγ2 Fw	TCTTGAGGAACCTGAGCAGAA	AM168523
IFNγ2 Rv	TGTGCAAGTCTTTCCTTTGTAG	
IL-17A/F2 Fw	ATGTCCTGATAAATGGG+CAGTGAG[a]	HM231140
IL-17A/F2 Rv	TGTCCTGATAAATGGGCAGT+GAGT[a]	

[a] The '+'is before the nucleic acid in which the locked nucleic acid bond was placed

Western blot analysis

TLR2-transfected HEK 293 cells were used to investigate protozoan parasite ligands using phosphorylation detected with an antibody specific for phospho-p38 as a measure for responsiveness. To this purpose, HEK 293 cells were resuspended by pippeting and transferred to pre-cooled eppendorf tubes. Cells were washed twice in ice-cold PBS, lysed on ice with lysis solution [0.5% Triton X-100, 20 mM Tris, 100 mM NaCl, 1 mM EDTA, 50 mM NaF (Sigma), 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma)], homogenized

with a syringe and incubated 10 min on ice. Cell lysates were centrifuged at 21000g for 10 min at 4 °C. Supernatant was collected and total protein content was determined by the Bradford method. Samples (20-25 µg) were boiled at 96 °C for 10 min with loading buffer containing β-mercaptoethanol and separated by 10% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes (Protrans, Schleicher & Schuell, Bioscience GmbH). Membranes were blocked in 3% BSA in TBS (10 mM Tris, 150 mM NaCl, pH 7.5) for 1 h at room temperature and then incubated with primary antibody overnight at 4 °C in 3% BSA in TBS. Following antibodies reactive to both humans and carp were used: rabbit IgG anti-phospho-p38 (1:1000, Thr180/Tyr182, BioCat GmBh, Heidelberg, Germany) and rabbit IgG anti-β-tubulin (1:500, Abcam, Cambridge, UK). Membranes were then incubated with goat-anti-rabbit HRP-conjugated (1:1000, Dako) in 10% milk powder in TBS for 1 h at room temperature. Between each incubation step, membranes were washed twice with TBS-Tween/Triton (TBS, 0.05% (v/v) Tween 20, 0.2% (v/v) Triton X-100) and once with TBS, for 10 min at RT. Signal was detected by development with a chemoluminescence kit (Amersham) according to the manufacturer's protocol and visualized by the use of Lumni-fil chemiluminescent Detection Film (Roche, Woerden, The Netherlands). The blots were scanned and saved as a greyscale TIFimages. Each image was converted to a binary image and the number of pixels in each band were quantified using a user-defined threshold in a custom-written MATLAB (release 2009a) script.

Statistical Analysis

Transformed values (ln) were used for statistical analysis in SPSS software (version 17.0). Homogeneity of variance was analyzed using the Levene's test. Significant differences ($P \leq 0.05$) for the *in vivo* gene expression studies were determined by a two-way ANOVA followed by a Sidak's test. Significant differences between treatments ($P \leq 0.05$) for the *ex vivo* and *in vitro* gene expression studies were determined by one-way ANOVA followed by Sidak's test. In case of unequal variances between treatments, the one-way ANOVA was followed by a Games–Howell test.

RESULTS

Live protozoan parasites induce TLR2 gene expression in carp macrophages

Glycosylphosphatidylinositol (GPI) anchors (or their fragments) from protozoan parasites have been shown to trigger TLR2 activation (36), (37). We made use of the fact that bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) can cleave GPI-anchors in eukaryotic cells. The cleavage promotes the release of soluble proteins into a supernatant now containing free GPI-phospholipids and diacylglycerol. The supernatant was used to study the possibility that GPI-anchored proteins from *T. borreli* and *T. carassii* can act as PAMPs of carp TLR2. Carp macrophages, when stimulated with supernatant from PI-

PLC-treated *T. borreli* protozoan parasites as a source of GPI-anchors, show a 2-3 fold up-regulation of TLR2 gene expression (18). GPI-anchors from another protozoan parasite of carp, *T. carassii*, also induced a dose-dependent up-regulation of TLR2 gene expression (Table II). Negative controls including non-PI-PLC-treated parasites and pellets from PI-PLC-treated parasites did not significantly modulate gene expression of TLR2 (data not shown). *In vitro* stimulation with live *T. borreli*, and to a lesser extent live *T. carassii*, resulted in an approximate 2 fold up-regulation of TLR2 gene expression in carp macrophages.

Table II. TLR2 fold change in carp macrophages after 6h stimulation with live or PI-PLC-treated parasites

	T. borreli [a]	***T. carassii*** [a]
PI-PLC-treated (millions)		
1	1.73	1.21
2.5	1.63	1.28
5	2.91*	1.44*
Live (millions)		
0.1	1.26	0.90
0.25	1.50	1.26
0.5	1.92	1.55

[a] Averages of four fish are shown.
* Signficant ($P \leq 0.05$) difference in gene expression compared to unstimulated cells. mRNA levels of TLR2 were normalized against the house keeping gene 40S ribosomal protein S11 and are shown as fold change relative to unstimulated macrophages

Protozoan-derived PAMPs are ligands of carp TLR2

HEK 293 cells were used to investigate protozoan parasite ligands for carp TLR2 using MAPK-p38 phosphorylation as a measure for responsiveness. HEK 293 cells were transfected with carp TLR2 full length (TLR2 WT) or with carp TIR-domain truncated TLR2 (TLR2ΔTIR). MAPK-p38 phosphorylation in transfected HEK 293 cells was detected using an antibody specific for phospho-p38 (18). Stimulation of HEK 293 cells transfected with truncated TLR2 (TLR2ΔTIR) never increased MAPK-p38 phosphorylation (TLR2ΔTIR, Fig. 1) when stimulated with GPI-anchors from protozoan parasites (supernatant from PI-PLC-treated) nor when stimulated with live parasites.

This negative control thereby showed the unresponsiveness of the parental HEK 293 cells to the protozoan parasite derived PAMPs. In contrast, stimulation of TLR2 WT-transfected HEK 293 cells increased MAPK-p38 phosphorylation, both with a source of GPI-anchors from both parasites and with live (*T. carassii*) parasites as stimulants (TLR2 WT, Fig. 1 A, B). The magnitude of the response was different between the two protozoan parasites with GPI-anchors from *T. carassii* inducing a higher MAPK-p38 activation than GPI-anchors from *T. borreli*. Thus, transfection of HEK 293 cells with TLR2 WT established the ability of carp TLR2 to recognize protozoan parasite, in particular *T. carassii*-derived PAMPs.

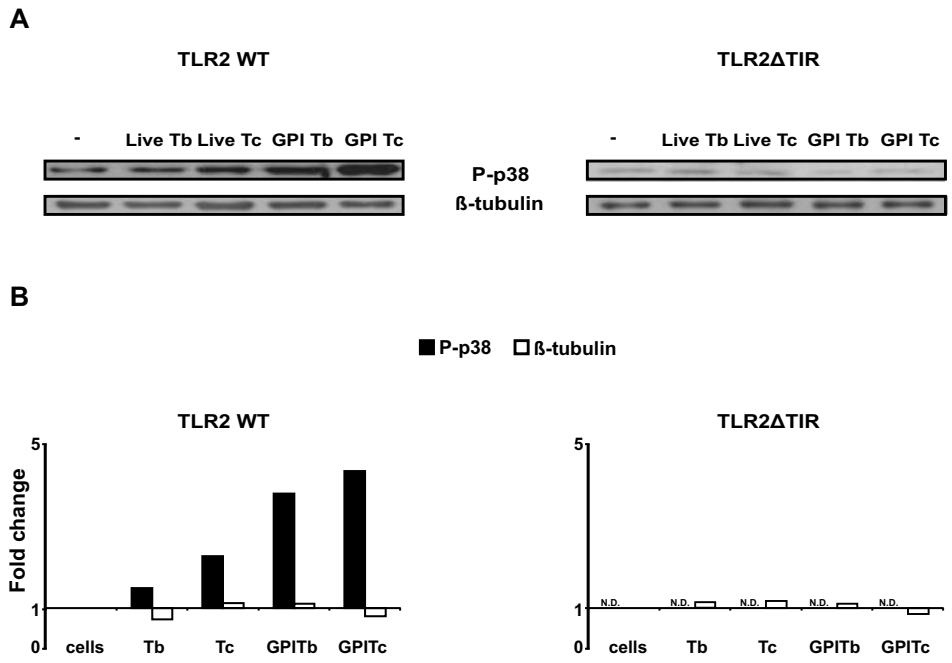

Figure 1. Activation of carp TLR2 by parasite-derived PAMPs in HEK 293 cells.
TLR2WT- and TLR2ΔTIR-transfected HEK 293 cells were stimulated with live *T. borreli* or *T. carassii* parasites (Live Tb or Live Tc, 0.5 million parasites per well), with supernatants from 5 million PI-PLC-treated- *T. borreli* or -*T. carassii* parasites (GPI Tb or GPI Tc) or left untreated as negative control. MAPK-p38 phosphorylation was analysed by immunoblotting for phospho-p38 (P-p38), while equal loading was confirmed by immunoblotting for β-tubulin (A). Fold change of P-p38 and β-tubulin protein levels in the stimulated cells of TLR2WT- and TLR2ΔTIR-transfected HEK 293 cells are shown relative to the untreated cells. The P-p38 signal intensity of TLR2ΔTIR-transfected HEK 293 cells was below the threshold and therefore non-detectable (N.D.) (B). One experiment representative of three independent experiments is shown.

Protozoan parasites induce or suppress different members of the IL-12 cytokine family

In mammalian vertebrates the IL-12 cytokine family comprises, among others, IL-12 (composed of p35 and p40 subunits) and IL-23 (composed of p19 and p40 subunits). For carp, a single p35 and three distinct p40 genes (named p40a, p40b and p40c) have been described (6). We cloned a partial cDNA of carp p19, comprising 255 nucleotides (GenBank accession number: HM231139) with 77 % sequence similarity to the zebrafish p19 gene (GenBank accession number: ACC77208) and designed primers for gene expression studies. We subsequently examined, in head kidneys of infected fish, changes in gene expression of p19, p35 and p40a-c during *in vivo* infection with two different protozoan parasites.

Infection with *T. borreli* did not induce significant changes in gene expression in none of the IL-12 cytokine family member subunits during the first two weeks of infection (8) as well as at later time points examined (Fig. 2A). In contrast, infection with *T. carassii* induced a significant 5 fold up-regulation of p40c (Fig. 2B), at least at 6 weeks post-infection.

To determine whether IL-12 cytokine family members were simply not regulated or selectively altered during protozoan infections, *ex vivo* recall responses to both parasites were performed. Head kidney leukocytes (HKL) from (non-)infected fish were stimulated *ex vivo* with PGN or PMA (positive controls), or with the homologous live parasite to assess the inducibility of the different IL-12 family members. In HKL from non-infected control fish, stimulation with PGN up-regulated p35, p40a, p40b, but not p40c nor p19. Stimulation with PMA induced a 5 fold up-regulation of p40c gene expression (data not shown). Neither PGN, nor PMA up-regulated gene expression of p19. In non-infected fish, stimulation with live parasites up-regulated p40b but not p19, p35, p40a nor p40c gene expression, independent of the protozoan parasite species. Thus, the recall responses of HKL of non-infected fish showed induced gene expression of the IL-12 family members p35 and p40, but not p19.

Ex vivo re-stimulation of HKL from infected fish with the carp TLR2 ligand PGN (18) provides information on the degree of (non)responsiveness acquired by HKL during infection. *Ex vivo* re-stimulation with PGN did not significantly increase p19 gene expression during *T. borreli* infection. In contrast, *ex vivo* re-stimulation with PGN increased p19 gene expression in cells from fish infected with *T. carassii* at week 3 and 5 of infection (PGN, Fig. 3 A and B). *Ex vivo* re-stimulation with PGN revealed a down-regulation of p35 gene expression in cells from infected fish, suggesting a suppression of expression of the p35 subunit during the whole period of *T. borreli* and *T. carassi* infections. *Ex vivo* re-stimulation with PGN increased p40a gene expression only in cells taken from fish early (1 week) during *T. borreli* infection, whereas in cells from fish infected with *T. carassii*, PGN increased p40a gene expression during the whole period of infection. *Ex vivo* re-stimulation with PGN showed that p40b gene expression was not influenced by infection with any of the two protozoan parasites, suggesting that p40b is not actively regulated during both protozoan parasite infections. *Ex vivo* re-stimulation with PGN (or with PMA) did not increase p40c gene expression in infected fish.

Ex vivo re-stimulation of HKL from infected fish with the homologous parasite provides information on the selectiveness of (non)responsiveness of the different IL-12 family members, acquired during infection (Fig. 3A and B). Re-stimulation of HKL with live *T. borreli* parasites significantly increased p19 gene expression (at week 5). Re-stimulation with *T. borreli* did not increase gene expression of p35 nor p40a. Gene expression of p40b or p40c was not affected by *T. borreli* infection (Live *T. borreli*, Fig. 3A). Re-stimulation of HKL with live *T. carassii* parasites significantly increased p19 (at week 3), but not p35 gene expression. Re-stimulation with live *T. carassii* parasites did not increase p40a gene expression, nor affected p40b gene expression. Re-stimulation of HKL with live *T. carassii* parasites, but not PGN, increased p40c gene expression at week 3 (Live *T. carassii*, Fig.

3B).

These results confirm the suppression of p35 gene expression during the whole period of protozoan infections and the propensity to up-regulate p19 gene expression at specific time-points of infection by both parasites. In addition, p40a gene expression was selectively impaired during *T. borreli* infection and never induced by *T. carassii* parasites. Re-stimulation of HKL with live parasites, similar to PGN, readily induced p40b gene expression, suggesting that p40b is not regulated during infection with protozoan parasites. In contrast, p40c gene expression in HKL could only be re-stimulated by live *T. carassii* parasites.

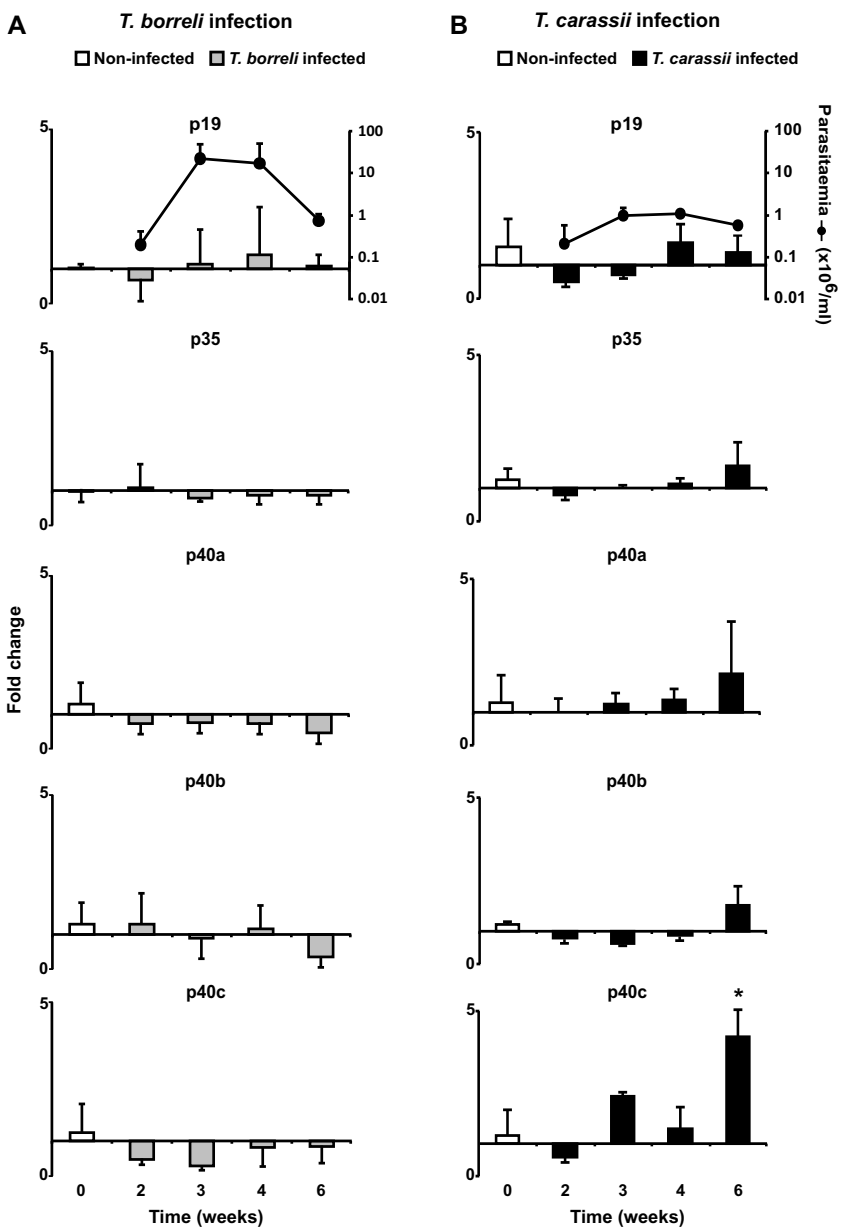

Figure 2. Kinetics of gene expression of IL-12 family members in head kidney during infection with *T. borreli* (A) or *T. carassii* (B). Carp were injected (i.p.) with a dose of 10000 parasites per fish, PBS-injected individuals served as negative controls (week 0). At indicated time points n=5 (*T. borreli*) or n=4 (*T. carassii*) animals were sacrificed for organ collection. Parasitaemia was monitored during infection and is shown in the upper plot, at a logarithmic scale. mRNA levels of IL-12 family members are shown relative to the house keeping gene 40S ribosomal protein S11. Data points represent averages + SD of n= 4-5 fish per time point. Symbol (*) represents a significant ($P \leq 0.05$) difference compared to non-infected fish.

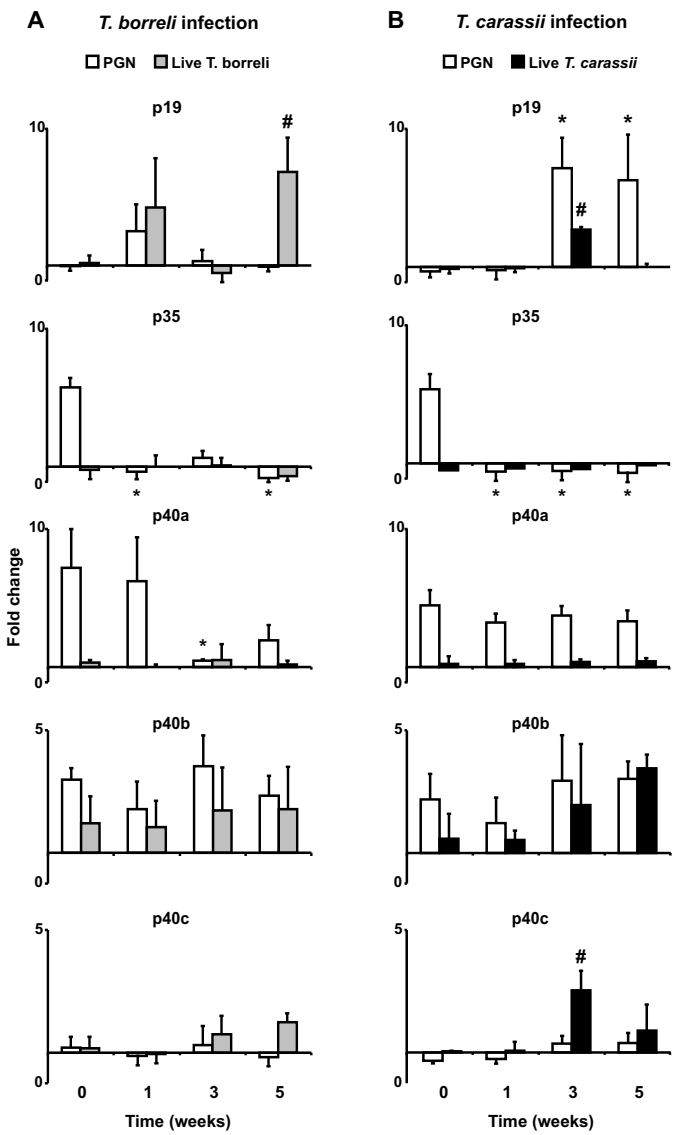

Figure 3. Kinetics of gene expression of IL-12 family members in re-stimulated head kidney leukocytes of *T. borreli*- (A) or *T. carassii*- (B) infected carp. Carp were injected (i.p.) with PBS or a dose of 10000 parasites per fish. Head kidney leukocytes (HKL) from non-infected (n=1) or infected fish (n=3) were isolated at different time points post-infection and re-stimulated with PGN (50 µg/mL) or homologous live parasites (0.25 million parasites per well) for 6h. mRNA levels of IL-12 family members were normalized against the house keeping gene 40S ribosomal protein S11 and shown as fold change relative to unstimulated head kidney leukocytes. Data points represent averages + SD of n=3 non-infected fish (taken at the three time points analysed and indicated as week 0) and n=3 infected fish per time point. Symbol (*) represents a significant ($P \leq 0.05$) difference between PGN re-stimulated HKL from infected fish compared to PGN re-stimulated HKL from non-infected fish. Symbol (#) represents a significant ($P \leq 0.05$) difference between parasite re-stimulated HKL from infected fish compared to parasite re-stimulated HKL from non-infected fish.

Regulation of gene expression of different IL-12 family members by live protozoan parasites is TLR2-mediated

Transfection of HEK 293 cells with TLR2WT established the ability of carp TLR2 to recognize protozoan parasite, in particular *T. carassii*-derived PAMPs (see Fig.1). To verify the association between TLR2 activation and downstream gene transcription of the different IL-12 family members, we overexpressed TLR2 in carp macrophages. Expression of both TLR2WT and TLR2ΔTIR constructs were confirmed after transfection by western blot using anti-GFP antibody (data not shown). Stimulation of macrophages overexpressing TLR2ΔTIR was used as negative control (TLR2ΔTIR, see Fig. 4). Stimulation of carp macrophages overexpressing TLR2WT with live *T. carassii* parasites could significantly increase p40b gene expression but not p35 nor p40a gene expression. In contrast, gene expression of the IL-12 family members p19 (P=0.06) and p40c was solely induced by live *T. carassii* parasites (TLR2WT, Fig. 4A and B).

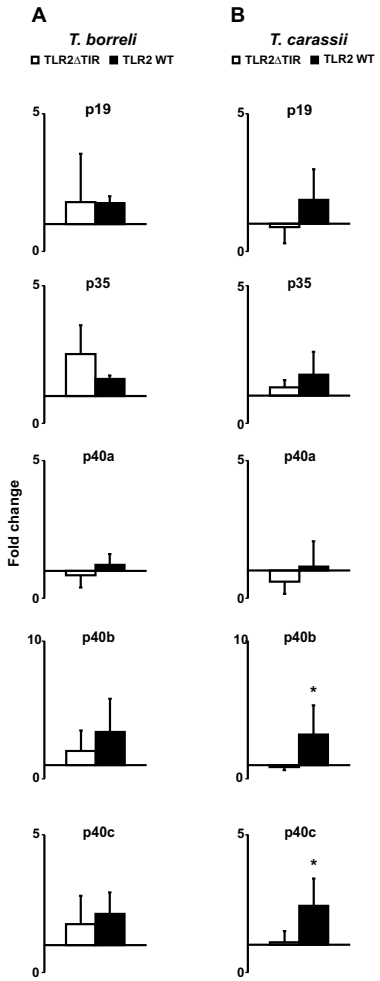

Figure 4. Overexpression of TLR2 in carp macrophages. TLR2ΔTIR- and TLR2WT-transfected carp macrophages were stimulated with live *T. borreli* or *T. carassii* parasites (0.25 million parasites per well) for 6h, or left untreated as negative control. mRNA levels of IL-12 family members were normalized against the house keeping gene 40S ribosomal protein S11 and are shown as fold change relative to unstimulated macrophages (fold change = 1). Bars show averages + SD of n=4 fish. Symbol (*) represents a significant ($P \leq 0.05$) difference in gene expression between parasite-stimulated cells macrophages in TLR2WT-transfected- compared to TLR2ΔTIR- transfected carp macrophages.

Transfection of HEK293 cells with TLR2WT showed that live *T. carassii* and GPI-anchors from *T. carassii* parasites can signal through carp TLR2. Overexpression of TLR2WT in carp macrophages confirmed a TLR2-mediated p19 and p40c regulation by the protozoan parasite *T. carassii.*

The protozoan parasite T. carassii, in particular, induces a Th17-like gene expression profile

Enhanced expression of the IL-12 gene family members p19 and p40c associated with *T. carassii* infections suggested this protozoan parasite, in particular, could induce an IL-23-like molecule in carp. The spleen is a secondary immune organ that increases considerably in size especially during *T. borreli* infections but also during *T. carassii* infections, indicative of an involvement in the immune response against these two parasites. To verify parasite-induced immune responses downstream of IL-23, we studied gene expression in the spleen of infected carp as a measure of a Th17-mediated immune response. Both parasites induced gene expression of the chemokines CXCb in spleen. However, infection with *T. carassii* also significantly induced CXCL8_L2 (a chemokine related to mammalian IL-8, (38)) in spleen leukocytes. Similar to the previous experiments, gene expression of p19 and p40c was significantly different between *T. borreli* and *T. carassii*-infected fish, with p40c being significantly up-regulated only in splenocytes of *T. carassii*-infected fish. Furthermore, *T. carassii* infections were characterized by an increase in gene expression of major histocompatility class II genes and the matrix metalloproteinase MMP-9 (Table III).

Table III: Gene expression of spleen leukocytes of parasite-infected carp relative to non-infected fish

Gene	Control[a]	*T. borreli*[a]	*T. carassii*[a]
IL-1β	1.11	1.19	2.80
TNF-α	1.22	2.02	2.28
p19[#]	0.84	0.84	1.61
p35	1.04	0.78	1.53
p40a	1.29	1.14	1.60
p40b	1.42	1.03	2.10
p40c[#]	1.05	1.17	7.02*
CXCa	1.19	1.26	1.47
CXCb	1.50	3.25*	3.08*
CXCL8_L2	1.94	4.37	4.29*
CXCR1	1.31	1.83	2.97
CXCR2	1.01	0.65	1.69
MHC-II DAB1-2	1.02	0.79	1.71*
MHC-II DAB3-4	1.01	1.16	1.80*
MMP-9	1.40	2.31	4.11*

[a] Averages (n=4) of 3-weeks *T. borreli* or *T. carassii* infected fish or non-infected fish (control) are shown.[*] Signficant difference compared to control. [#] Signficant difference between *T. borreli* and *T. carassii* infected fish. mRNA levels of the analysed genes were normalized against the house keeping gene 40S robosomal protein S11 and are shown as fold change relative to non-infected fish.

The protozoan parasite T. carassii induces neutrophilia in spleen

Up-regulation of gene expression of the IL-12 gene family members p19 and p40c, in combination with up-regulation of gene expression of chemokines (CXCb and CXCL8_L2) and of the matrix metalloproteinase MMP-9, suggested infections with *T. carassii* could be associated with a Th17-like immune response typically associated with neutrophilia. To investigate this hypothesis, splenic tissue from infected fish was stained for the presence of macrophages (WCL-15$^+$ cells) versus the presence of neutrophilic granulocytes (TCL-BE8$^+$ cells). Macrophages and neutrophilic granulocytes were present as scattered single cells in non-infected fish (Fig. 5A and 5B).

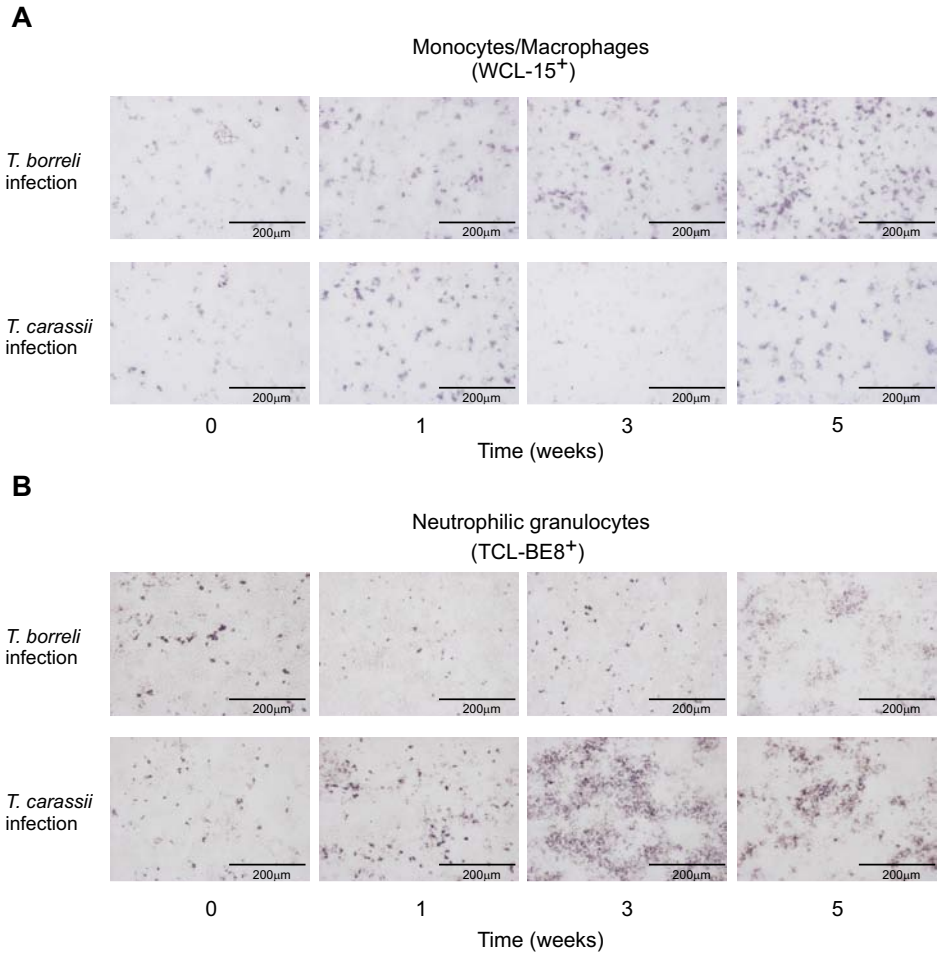

Figure 5. Macrophage (A) and neutrophilic granulocyte (B) cell populations in spleen of *T. borreli*- and *T. carassii*- infected fish. Carp were injected (i.p.) with a dose of 10000 parasites per fish, PBS-injected individuals served as negative controls. Spleens from non-infected and infected fish were collected at different time points post-injection. Monocytes/macrophages (WCL-15+) and neutrophilic granulocytes (TCL-BE8+) are stained in red. One experiment representative of three independent experiments is shown.

During infection with *T. borreli* a clear but moderate increase in the number of macrophages was observed from week 3 (peak of parasitaemia) of infection onwards whereas a moderate increase in the number of neutrophilic granulocytes was seen at weeks 5 post-infection only. In contrast, in *T. carassii*-infected fish, a decrease in the number of macrophages was observed at week 3 post-infection, whereas a striking increase in the number of neutrophilic granulocytes was observed, often as TCL-BE8^{+} aggregates, from week 1 onwards with a peak at 3 weeks post-infection.

Neutrophilic granulocytes from spleen of T. carassii-infected fish show a high p19 and p40c gene expression

The number of neutrophilic granulocytes markedly increased during *T. carassii* infection, as judged by histological examination. Splenic neutrophilic granulocytes (TCL-BE8^{+} cells) were collected from *T. carassii*-infected fish (week 3) and sorted to purity (>90%, assessed by flow cytometry) for measurement of constitutive gene expression levels of the IL-12 gene family members.

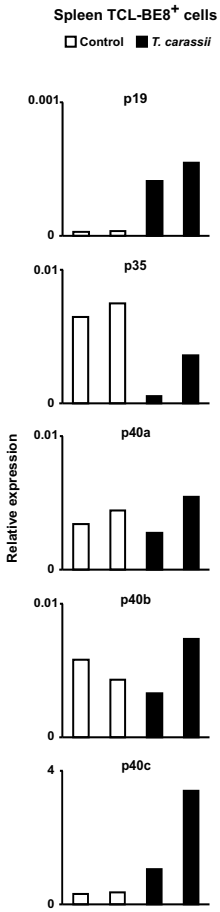

Figure 6. Constitutive gene expression of IL-12 family members in neutrophilic granulocytes from *T. carassii*- infected fish. Carp were injected (i.p.) with a dose of 10000 parasites per fish, PBS-injected individuals served as negative controls. Three weeks post-infection, splenocytes from non-infected (n=2) or *T. carassii*-infected fish (n=2) were isolated and neutrophilic granulocytes (TCL-BE8+ cells) sorted to purify by magneting sorting. Each bar represents a single fish.

Splenic neutrophilic granulocytes from *T. borreli*-infected fish were not altered at week 3 and not examined. For most of the IL-12 cytokine family genes, the level of gene expression in neutrophilic granulocytes isolated from spleen of infected fish was comparable to that seen in spleen of non-infected fish (Fig. 6). However, a high constitutive expression level of the p19 and p40c genes was observed in splenic neutrophilic granulocytes from *T. carassii*-infected fish when compared to non-infected fish.

IFN-γ and IL-17A/F gene expression are differentially regulated during T. borreli and T. carassii infections

In *T. carassii*-infected fish, up-regulation of gene expression for CXCb and CXCL8_L2 chemokines coincided with a marked increase of neutrophilic granulocytes in the spleen. Chemokine-dependent neutrophil recruitment, promoted by IL-17A/F, is characteristic of Th-17-mediated immune responses. For carp, two IFN-γ genes have been described, of which IFN-γ2 is associated with T cell responses (21). We cloned a partial cDNA of carp IL-17A/F2 (GenBank accession number: HM231140), comprising 173 nucleotides with 90% sequence similarity to the zebrafish IL-17A/F2 gene and 11 and 13% sequence similarity to the zebrafish IL-17A/F1 and IL-17A/F3 genes, respectively (GenBank accession numbers: NP_001018623 [A/F1] NP_001018634 [A/F2] NP_001018626 [A/F3]) and designed primers for gene expression studies. To evaluate the development of a Th1- or Th17-mediated immune response during protozoan parasite infection of carp, we measured IFN-γ2 and IL-17A/F2 gene expression, respectively. Gene expression studies in PBL showed an upregulation of IFN-γ2 gene expression in PBL of *T. borreli*-infected fish that correlated with parasitaemia, with a peak at 3 weeks post infection. In contrast, upregulation of IFN-γ2 gene expression in PBL of *T. carassii*-infected fish was observed only at a late time point (week 5) (Fig. 7A). Transcription of IL-17A/F2 was undetectable in PBL (data not shown). Gene expression studies in head kidney and spleen showed an up-regulation of IFN-γ2 gene expression in both organs from *T. borreli*-infected fish, but only in head kidney from *T. carassii*-infected fish. Transcription of IL-17A/F2 was not regulated in immune organs of *T. borreli*-infected fish. In contrast, a significant increase of IL-17A/F2 gene expression was observed in head kidney of *T. carassii*-infected fish (Fig. 7B).

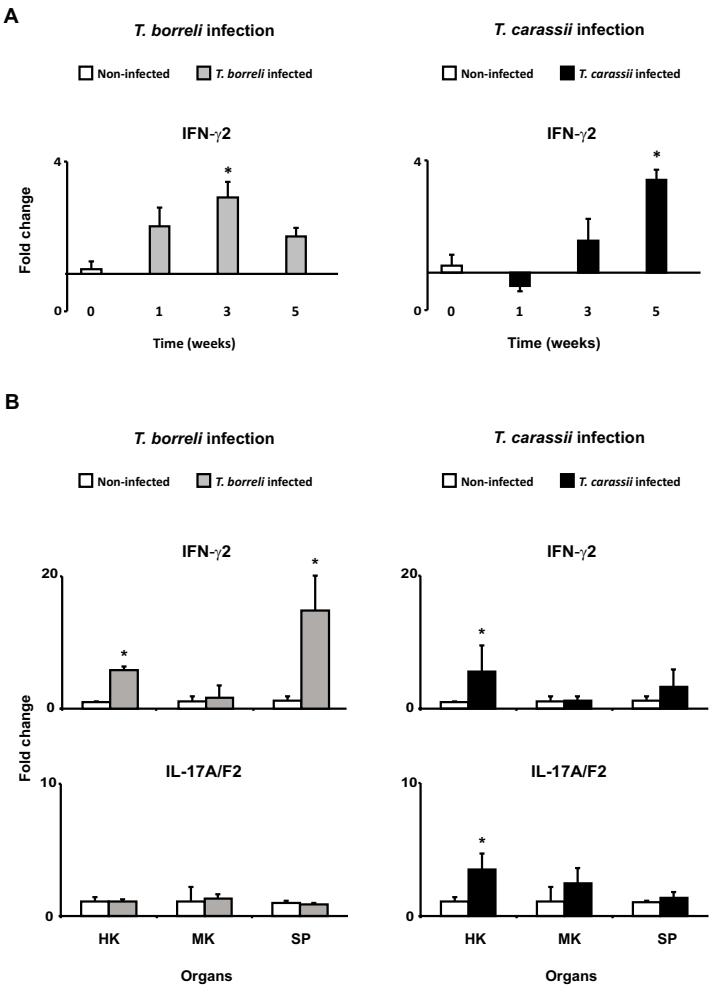

Figure 7. IFN-γ2 and IL-17A/F2 gene expression during *T. borreli*- and *T. carassii*-infection.
Gene expression was measured at different time points post-infection in peripheral blood leukocytes (PBL) (A) or at a fixed time point (3 weeks) in head kidney (HK), mid kidney (MK) and spleen (SP) (B). Carp were injected (i.p.) with a dose of 10000 parasites per fish, PBS-injected individuals served as negative controls. mRNA levels of IFN-γ2 and IL-17A/F2 were normalized against the house keeping gene 40S ribosomal protein S11 and expressed as fold change relative to non-infected fish. Data points represent averages + SD of n= 3 non-infected fish (indicated as week 0) and n=3 infected fish per time point. Symbol (*) represents a significant ($P \leq 0.05$) difference compared to non-infected fish.

DISCUSSION

In our previous studies on the immune response of carp to protozoan parasites, we have measured a characteristically high production of NO by classically activated macrophages

('type 1 response') induced by *T. borreli*. Classically activated macrophages are antagonized by type 2 anti-inflammatory responses, which are driven by the development of alternatively activated macrophages. In our previous studies we have measured a preference for an alternative activation of macrophages ('type 2 response') induced by *T. carassii* (24). Thus, these two protozoan parasites, though both extracellular blood parasites, induce different immune responses in the carp host. In the present study we have refined our investigations and conclude that infections with *T. carassii*, in particular, are associated with a Th17-like immune response. In support of this conclusion are: 1) simultaneous up-regulation of p19 and p40c gene expression in *T. carassii*-restimulated HKL and in carp TLR2-transfected macrophages stimulated with *T. carassii*, suggestive of the formation of the cytokine IL-23, 2) a strong neutrophilia in the spleen of *T. carassii*-infected fish, coinciding with the presence of chemokines and metalloproteinase MMP9, leading to a high number of neutrophils that express p19 and p40c, 3) induction of IL-17A and IL-17F, as suggested by the up-regulation of IL-17A/F2 gene expression in head kidney of *T. carassii*-infected fish.

Several studies in mammalian vertebrates have shown the involvement of TLR2 in recognition of GPI-anchors derived from protozoan parasites (39) including *Leishmania* spp (40), *T. gondii* (41), *T. cruzi* (36) and *P. falciparum* (42). In our studies, the carp TLR2 receptor, when transfected into human cells (HEK 293) mediated the recognition of protozoan parasite-derived PAMPs. Similar to mammalian vertebrate TLR2, carp TLR2 recognizes both live protozoa as well as protozoan-derived GPI anchors, especially GPI anchors from *T. carassii*, and triggers phosphorylation of MAPK-p38 (this study). Overexpression of TLR2 in carp macrophages not only confirmed recognition of protozoan PAMPs by TLR2 but also established a TLR2-mediated p19 and p40c (IL-23) up-regulation by the protozoan parasite *T. carassii*. In mammalian vertebrates, the balance between different IL-12 cytokine family members (IL-12, IL-23 and IL-27) during the course of an immune response can be differentially regulated downstream of particular pattern recognition receptors, such as the TLRs (17). For example, TLR2 activation by peptidoglycan from Gram-positive bacteria potently induces p19 and thus IL-23 synthesis (15). Provided that, also in carp, p19 gene expression correlates with the production of IL-23, our data suggest there is a link between activation by protozoan PAMPs of the TLR2 receptor on, for example carp macrophages, and downstream production of the cytokine IL-23.

The cytokine IL-23 is composed of two subunits; p19 and p40. Recently, the cDNA sequence of p19 of zebrafish, a close relative of carp was described (9). We designed primers for gene expression studies on a carp p19 cDNA sequence. Three distinct p40 genes (p40a, p40b and p40c) had already been reported for carp (6). The identification of multiple p40 genes in carp increases the potential for heterodimeric combinations between p19, p35 and p40 subunits (43). It is difficult to unambiguously identify the most likely candidate, if any, of the three p40 isoforms for heterodimerization with p19 to form carp IL-23. Three-dimensional modeling based on sequence information pointed at p40a and p40b, more than the p40c isoform, as likely candidates for heterodimerization with p35, to form IL-12

(6). Although three-dimensional modeling does not provide evidence for the p40c isoform to preferentially heterodimerize with p19 to form IL-23, the simultaneous up-regulation of gene expression of p19 and p40c in *T. carassii*-re-stimulated head kidney leukocytes and in carp TLR2-transfected macrophages stimulated with *T. carassii*, suggests that heterodimerization of the p40c subunit with p19 could preferentially lead to formation of the cytokine IL-23 in carp. Of note, the constitutive gene expression of p40c is much higher than that of the other two p40 isoforms. Despite the high constitutive expression, p40c gene expression is still inducible. This suggests that high levels of p40c proteins could be produced, maybe leading to the formation of p40c homodimers that could bind to the IL-12 receptor and behave as antagonists of IL-12 (p35, p40)-mediated responses, as observed in mammals (44); (45). For certain, future studies, for example with plasmids composed of the different p40 subunits fused to the p19 subunit, are required to help identify the bio-active carp IL-23 protein.

The cytokine IL-12 constitutes an important factor in the differentiation and expansion of Th1 cells and plays a pivotal role in the production of IFN-γ, which in turn activates anti-microbial activity in effector cells (10). For that reason it can be beneficial for particular microbes to specifically suppress IL-12 (p35, p40) production. The ability of protozoan parasites to suppress IL-12 formation has been documented for leishmaniasis (46), (47) in mice and trypanosomiasis in rats (48). We found that both protozoan parasites of carp have the ability to suppress the induction of p35 and p40a gene expression gene expression during the whole period of infection, but neither parasite affected p40b gene expression (see fig. 3). This could indicate that the formation of an IL-12 molecule by heterodimerization of p35 and p40a would be the prime target for inhibition by these protozoan parasites of carp. The constitutive gene expression of p35 (and of p19) is low compared to the constitutive gene expression of p40a-c, which could mean that minimizing p35 (or p19) expression could be enough to restrict IL-12 (or IL-23)-dependent immune responses. In the case of heterodimerization of p35 and p40b, suppression of gene expression of p35 only, could be sufficient to inhibit formation of IL-12. Despite the apparent absence of IL-12 (p35) expression during both protozoan infections in carp, we observed induction of IFN-γ2 gene expression during *T. borreli* and (to a lower extent) during *T. carassii* infection. Innate immune cells such as (natural killer) NK cells, NKT cells, γδT cells or the effector Th1 or CD8+ T cells may be responsible for the observed IFN-γ2 production (49), (50).

The development of Th17 cells from naïve T cells is dependent on antigen presentation, co-stimulatory stimulation and a specific cytokine milieu (e.g.: IL-1β, IL-6, TGF-β) with IL-23 production being important for the maintenance of Th17 cells (51). Both IL-17A and IL-17F cytokines produced by active Th17 cells appear to be especially potent activators of neutrophils, both through expansion of the lineage through regulation of G-CSF and G-CSF receptor as well as through recruitment of high numbers of neutrophils by chemokines such as CXCL1, CXCL2 and CXCL8 (52), (53). Studies in mice revealed that ectopic expression of IL-17 stimulates a strong neutrophilic response whereas IL-

17 deficiencies are associated with neutrophil defects leading to disease susceptibility (52), (54). Thus, the Th17-mediated immune response is characterized by an IL-17A/F-driven increase of chemokines and metalloproteinases associated with recruitment and activation of neutrophils (53). Infection of carp with the two different protozoan parasites induced different immune responses. Infection with *T. borreli* lead to an increase of IFN-γ gene expression and a moderate increase in the number of macrophages in the spleen. In contrast, infection with *T. carassii* lead to an increase of CXCb (CXCL9-11-like chemokine), CXCL8_L2 (IL-8-like chemokine) and MMP-9 gene expression and a marked increase in the number of neutrophilic granulocytes in the spleen. The concomitantly high p19 and high p40c gene expression in these neutrophils suggests a role for these cells in maintaining an IL-23-dependent immune response. Chemokine-dependent neutrophil recruitment suggests that the carp host responds to infections with *T. carassii* with a Th17-mediated immune response.

The cytokine IL-23 is required for the expansion of Th17 cells, which produce IL-17A and IL-17F but also IFN-γ (55),(13). Three distinct IL-17A/F cDNA sequences have been identified in zebrafish (14). We designed primers for gene expression studies on a carp IL-17 cDNA sequence. The cytokine IFN-γ exists in carp as two distinct genes with IFN-γ2, in particular, associated with characteristic T-lymphocyte function (21). During infections with *T. borreli*, we observed an up-regulation IFN-γ2, but not of IL-17A/F2, in head kidney and spleen. In contrast, in *T. carassii*-infected fish, we observed an up-regulation of both IFN-γ2 and IL-17A/F2 in head kidney. In mammalian vertebrates, several studies indicate the presence of subsets of cells producing both IFN-γ and IL-17A/F. Double producers may represent a transitional state between Th17 and Th1 (13), (56) . Furthermore, in mice it has been shown that Th17 are phenotypically unstable and readily convert to a Th1 phenotype, but not vice versa (57), (58). When transferred to Th1-polarizing conditions and in the absence of IL-23, Th17 cells decrease synthesis of the IL-23R chain, thus allowing the reconstitution of the heterodimeric IL-12 receptor and promoting the production of IFN-γ. It has been suggested that T cells either co-produce IFN-γ and IL-17A/F or suppress IL-17A/F and continue to produce IFN-γ (13). The co-induction of IFN-γ2 and IL-17A/F2 during infection with *T. carassii* suggests the presence of mixed Th1/Th17 phenotypes in *T. carassii*-infected fish. Although host defense against extracellular bacteria is widely considered to be dominated by a Th17 response (59), a mixed Th1/Th17 polarization was observed during *Bordetella pertussis* infection (60). The fact that IFN-γ2 is induced especially at later stages of the *T. carassii* infection suggests that carp IFN-γ could play a role in limiting Th17-mediated responses (61). Therefore, efficient protection against the extracellular protozoan parasite *T. carassii* in carp may require synergy between Th17- and Th1-mediated cell responses.

In mammalian vertebrates, IL-12 is important for the differentiation and expansion of Th1 cells and production of IFN-γ against intracellular bacteria and parasites. IL-23 is important for the expansion of Th17 cells, which produce IL-17 in response to extracellular bacteria and fungi. There is some evidence regarding the role of Th17-mediated responses

to protozoan parasites, but it is limited. Infection of IL-17-deficient mice with *Toxoplasma gondii* lead to a higher mortality than in control mice (62). In contrast IL-27, which suppresses Th17 responses, was beneficial for the host during *Trypanosoma cruzi* infection (63). There are several studies that implicate TLR2 activation in p19 gene expression (64), neutrophil transmigration (65) and promotion of Th17 responses (66). Our studies show that *T. carassii*-derived PAMPs are agonists of carp TLR2, promoting p19 and p40c gene expression. This is the first study that provides evidence for a Th17-like immune response in fish to infection with protozoan parasites.

ACKNOWLEDGMENTS

We thank the central facilities, "De Haar-Vissen", for taking care of the carp. We also thank Prof. N. Okamoto for providing the mouse mAb TCL-BE8 and Maria Forlenza for providing the samples from the *in vivo T. borreli* experiment. Anja J. Taverne-Thiele, Trudi Hermsen and Sander Kranenbarg are gratefully acknowledged for technical support.

FUNDING

This work was supported by the Portuguese foundation Fundação para a Ciência e Tecnologia under Grant agreement number SFR/BD/21522/2005 and the European Commission through contract FP6007103 (Improved Immunity of Aquacultured Animals, IMAQUANIM).

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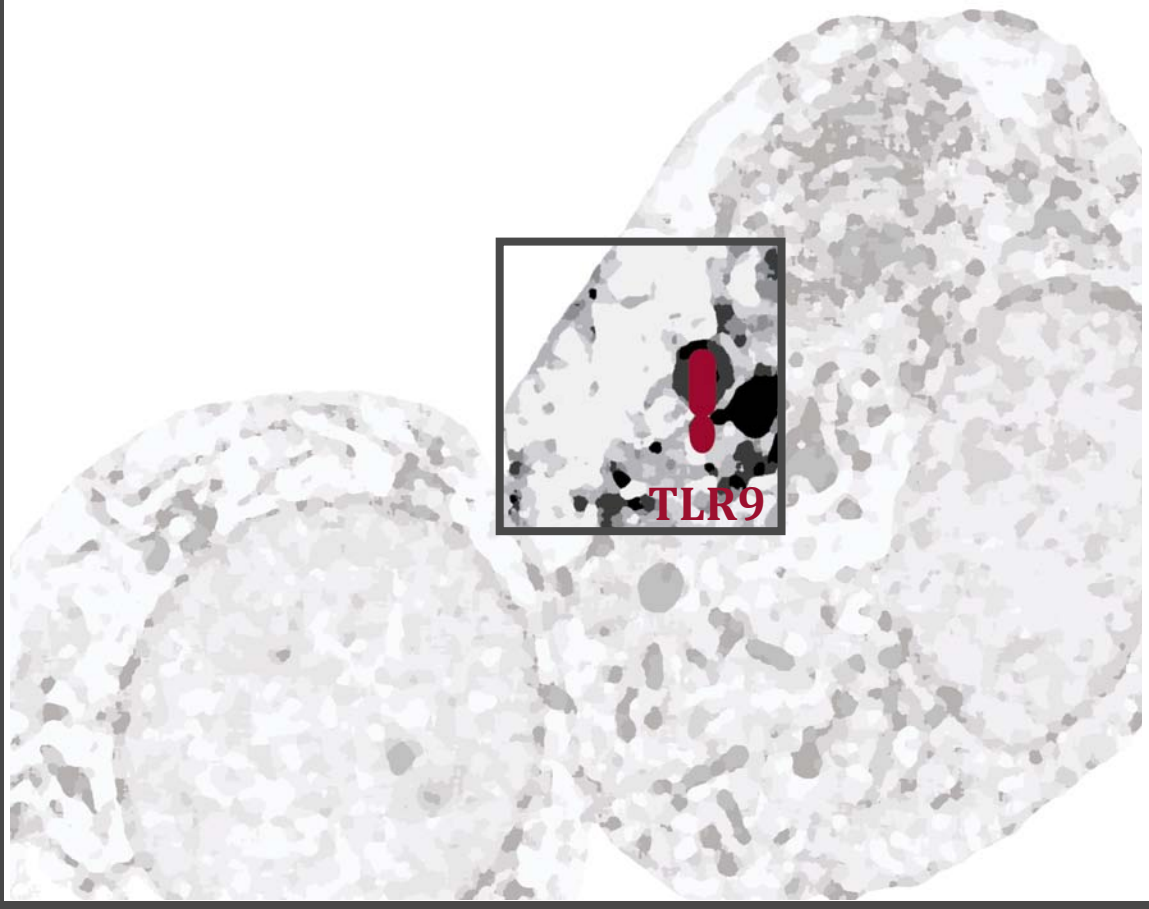
“ Times change, Wills change. ”

Luis Vaz de Camões



Chapter 6

Recognition of bacterial DNA: indications for a protease-dependent route of activation for carp TLR9

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Manuscript in preparation

ABSTRACT

Mammalian vertebrate TLR9 has been grouped, together with TLR7 and TLR8 in the TLR7 subfamily. Carp TLR9 has a TLR7 family signature with 26 leucine-rich repeats, a super-motif TTSTTSTT in the N-terminal region and an extended flexible loop in LRR15. A longer extended flexible loop is predicted for carp as well for the other fish species when compared to the mammalian TLR9. Carp TLR9, when transfected into human HEK cells, recognizes the prototypical TLR9 ligand bacterial *E. coli* DNA, but not the protozoan *T. borreli* DNA or any of the selected phosphorothioate-modified CpG ODNs. Stimulation of carp macrophages with bacterial DNA resulted in a strong cell activation as shown by cytokine gene expression, nitrogen radical production and MAPK-p38 activation. Inhibition of protease activity in carp macrophages decreased cytokine (IL-1β and p35) gene expression following stimulation with bacterial DNA. Our study suggests the presence of protease-dependent TLR9 activation route for carp TLR9.

INTRODUCTION

Macrophages respond to pathogens by recognizing conserved pathogen-associated molecular patterns (PAMPs). The activation of macrophages by PAMPs is profoundly mediated by the TLR family of pattern recognition receptors. The engagement of PAMPS by TLRs results in the activation of intracellular signalling pathways initially mediated through the recruitment of adaptor molecules such as MyD88. Downstream activation of protein kinases, such as IκB kinase and MAPKs ultimately leads to the expression of proinflammatory genes such as IL-1β, TNF-α and IL-12 and to the production of radicals (1)(2).

TLR9 recognizes DNA containing unmethylated CpG motifs such as viral and bacterial (e.g. *Escherichia coli*) DNA that contain short sequences of unmethylated CpG dinucleotides in higher frequency than eukaryotic DNA, where CpG motifs are suppressed and mostly methylated (3-5). Studies into CpG recognition by TLR9 often make use of synthethic, nuclease-resistant and therefore more stable phosphorothioate-modified oligodeoxynucleotides (PS-ODN). This PS modification also greatly enhances uptake of the ODNs by macrophages (6, 7). Studies in human and mice have classified the CpG ODNs into three different types (A, B, C) based on their structural, organization and CpG content and therefore their biological differences (8). In humans, plasmacytoid dendritic cells and B cells have been identified as primary target for CpG ODNs (9). In mice, macrophages are the prime cell type stimulated by B-type CpG ODNs (10). TLR9 orthologues have been identified in several teleost fish Orders (Cypriniformes (11), Perciformes (12), Pleuronectiformes (13), Salmoniformes (14). In addition, studies in representatives of these Orders (e.g. Atlantic salmon (15), rainbow trout (16), common carp (17)) have reported immunostimulatory

activities for CpG ODNs of the A, B or C class using cytokine gene expression, cell proliferation and radical production as read-out. However, until present, for no fish species has a clear proof been published that directly links the presumed TLR9 ligand (e.g. pathogenic DNA, CpG ODNs) to the presence of the TLR9 protein.

In mammalian vertebrates, a cellular activation response to CpG motifs requires trafficking of TLR9 from the endoplasmic reticulum (ER) to the endolysosomes that contain the exogenous DNA (18). The subcellular localization of TLR9 has been suggested crucially important for the discrimination between self and non-self nucleic acids, avoiding inappropriate activation by self nucleic acids (19). Recent reports revealed that the presence of a truncated form of TLR9 is restricted to the endolysosomes of unstimulated cells (20). It has been shown that the full-length TLR9 is sorted to the ER, traffics through the Golgi and is routed to the endolysosome, where it is cleaved by resident proteases (21). The truncated form of TLR9 can also bind to CpG ODNs and initiate signaling cascades mediated by the recruitment of the adaptor molecule MyD88. Although there are conflicting data regarding the specific proteases responsible for the cleavage of TLR9, cysteine proteases appear to be involved in the processing of TLR9 (22).

European common carp is the natural host of the eukaryotic protozoan parasite *Trypanoplasma borreli* (23). It has been previously shown that DNA from *T. borreli*, although at high concentrations of 50 µg/ml, induced nitric oxide (NO) production in carp head-kidney phagocytes. DNAse-treatment and cytosine methylation treatment lowered the production of NO, suggesting that CpG motifs in *T. borreli* DNA could stimulate carp phagocytes (24). We analyzed the TLR9 receptor sequence of European common carp. Three-dimensional modelling confirmed the presence of 26 leucine-rich repeat (LRR) regions predicted by sequence analysis. Transfection of human (HEK 293) cells with carp TLR9 confirmed the ability of *E. coli* DNA, but not PS-ODNs nor *T. borreli* DNA, to trigger TLR9-dependent MAPK-p38 activation. Three-dimensional modelling confirmed the presence of a flexible loop on LRR15 predicted by sequence analysis. Inhibition of protease activity in carp macrophages lowered cytokine activation in these cells, which is downstream of the presumed recognition of *E. coli* DNA by the macrophage. This suggests that carp TLR9 may be cleaved by host proteases in a similar way as reported for TLR of mammalian vertebrates. We discuss an evolutionary conservation of protease-dependent TLR9 activation in carp.

Material and Methods

Animals

European common carp (*Cyprinus carpio carpio* L.; in this study refered to as 'carp' unless stated otherwise) were reared in the central fish facility of Wageningen University, The Netherlands at 23 °C in recirculating UV-treated tap water and fed pelleted dry food (Skretting, Nutreco) daily. R3xR8 carp are the hybrid offspring of a cross between fish of

Hungarian origin (R8 strain) and of Polish origin (R3 strain) (25). Carp were between 9 and 11 months old at the start of the experiments. All studies were performed with approval from the animal experimental committee of Wageningen University.

Trypanoplasma borreli DNA

Trypanoplasma borreli was cloned and characterized by Steinhagen at al. (23). Parasites were maintained by syringe passage through carp. Parasitaemia was monitored in 10x diluted blood in cRPMI [RPMI 1640 (Invitrogen, CA, USA) adjusted to carp osmolarity 280 $mOsmkg^{-1}$ containing 50 U/ml of heparin (Leo Pharma BV, Weesp, The Netherlands)] using a Bürker counting chamber. The minimum detection limit by this method was 10^5 parasites/ml of blood. For parasite isolation, blood was collected from 3-weeks-infected fish and purified on a 1x12cm ion-exchange chromatography using DEAE cellulose (DE-52; Whatman international) (26). After purification, $2x10^7$ parasites were pelleted and parasite DNA isolated using the DNeasy kit catalog no: 69504 (Qiagen, Leusden, The Netherlands) according to the manufacturers´ protocol.

Stimuli and inhibitors

Synthethic type-A CpG PS-ODN 2216 [5' GGGGGACGATCGTCGGGGGG-3']; type-B CpG PS-ODN 1681 [5'-ACCGATGTCGTTGCCGGTGACG-3'] and type-C CpG PS-ODN 2395 [5'-TCGTCGTTTTCGGCGCGCGCCG-3'] and the non-CpG control [5'–GGGGGATGCCTGGGGGG-3'] were purchased from Eurogentec (Eurogentec S.A., Seraing, Belgium). Synthethic type-B CpG ODN 1668 [5'-TCCATGACGTTCCTGATGCT-3'], synthetic type-B CpG ODN 2006 [5'-TCGTCGTTTTGTCGTTTTGTCGTT-3'], single-sheared *Escherichia coli* DNA complexed with the lipid-based transfection reagent LyoVec (*E. coli* ssDNA/LyoVec) and Z-VAD-fmk (carbobenzoxy-valyl-analyl-aspartyl-[O-methyl]-fluoromethylketone) were purchased from InvivoGen (Cayla SAS, France). Pepstatin A was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Amplification of carp TLR9 full-length cDNA

At the time of these experiments, the coding sequence for common carp TLR9 was unknown. Only recently, a cDNA sequence for common carp was published (GenBank Accession no: ADE20130.1, released 31st March 2010). Oligonucleotide primers for carp TLR9 were designed based on known partial zebrafish TLR9 sequences. cDNA from macrophages stimulated with CpG ODN was used as template for PCR. Based on partial carp cDNA sequences, new gene-specific primers (Table I) were designed to obtain the full-length carp TLR9. The 5´and 3´ends of carp TLR9 were amplified using gene-specific primers by 5´and 3´ rapid amplification of cDNA ends (RACE) (27) using the Gene Racer™ RACE Ready cDNA kit (Invitrogen, Breda, The Netherlands) according to the manufacturer´s protocol. First, gene-specific primers were used in combination with the Gene Racer primers to amplify the first strand of cDNA. A second round with gene-specific primers was performed to obtain the carp full-length TLR9 (TLR9, 3195 bp). PCR reactions were performed in *Taq* buffer, using 1U *Taq* polymerase (Promega, Leiden, The Netherlands) supplemented with

$MgCl_2$ (1.5 mM), dNTPs (200 µM) and primers (400 nM each) in a total volume of 50 µl. PCR and anchored PCR were carried out under the following conditions: one cycle 2 min at 96 °C; followed by 35 cycles of 30 sec at 96 °C, 30 sec at 55 °C and 3,5 min at 72 °C; and final extension for 7 min at 72 °C, using a GeneAmp PCR system 9700 (PE Applied Biosystems, Foster City, CA). Products amplified by PCR, or anchored PCR were ligated and cloned in JM-109 cells using the pGEM-Teasy kit (Promega) according to the manufacturer´s protocol. From each product both strands of eight clones were sequenced, using the ABI prismBigDye Terminator Cycle Sequencing Ready Reaction kit and analysed using 3730 DNA analyser. The TLR9 cDNA sequence reported in the present study comes from RNA isolated from European common carp, *Cyprinus carpio*, subspecies *carpio*.

Table I: Gene-specific primers used in RACE PCR

Primer	Sequence (5'-3')
TLR9 Fw1	CTT GAA GAC CAT TCC TCC TCT ACC
TLR9 Fw2	AAC TCA AAA AGT CAT CCT ACC GAC CTC
TLR9 Fw3	GA GAA TGG ACT CTA ATG TGC TTA GTT AT
TLR9 Rv1	TAA ATT CCA GTC CCA GAA TCT CAA GC
TLR9 Rv2	GTC GGT AGG ATG ACT TTT TGA GTT TGC
TLR9 Rv3	CAC ACT ACC AAG GAT GGA GAC ATA AAT GT

Bioinformatics

The carp TLR9 nucleotide sequence was translated using the ExPASy translate tool (http://us.expasy.org/tools/dna.html) and aligned with Clustal W (http://www.ebi.ac.uk/clustalw). The signal peptide cleavage site was predicted by using the SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) server. Identification of leucine-rich repeats (LRRs), transmembrane domain and Toll/IL-1 receptor (TIR) domain within carp TLR9 were predicted first, using the PFAM (http://pfam.sanger.ac.uk/) and SMART (http://smart.embl-heidelberg.de/) servers. Second, LRRs candidates that could not be recognized by PFAM, were identified by multiple sequence alignments with TLR9 from other species including human, mouse and zebrafish. Third, the protein secondary structure predictions of the LRR candidates were evaluated by the Proteus (http://wks16338.biology.ualberta.ca/proteus/) server.

Three-dimensional modeling of carp TLR9

The structure of mouse TLR3 ectodomain with a resolution of 2.66 Å (PDB entry: 3CIG) was used as a template to model the carp TLR9 using the programme MODELLER (version 9v8 (28)(29)) using the CVFF force field (30). The sequence of the loop region of carp TLR9 could not be modelled on the mouse TLR3 template because the TLR3 structure does not have the loop region. For this reason, the sequence coding for the loop region of carp TLR9 was removed from the initial TLR9 sequence used as input for the modelling. The carp TLR9 loop region was modelled separately using as template part of the transketolase structure from *Leishmania mexicana* (PDB entry 1L9J). Although the latter template was

chosen for highest sequence identity, the obtained three-dimensional structure for the loop region must be considered highly speculative. Also the N-terminal and C-terminal part were removed from the carp TLR9 sequence used for modelling, because the mouse TLR3 protein was crystallized without these regions.

TLR9-GFP and TLR2ΔTIR-GFP expression plasmids

RNA from macrophages stimulated with CpG ODN was used as template for PCR to clone the full-length carp TLR9. Two rounds of PCR were performed with the following primers: TLR9Fw-GCCATGGTTGGACACATGTTGTATCTG and TLR9Rv-ATGACACTCCAGGCATCCAGTC, using the product of the first round as template for the second round to obtain the full-length TLR9. PCR reactions were performed using the Long Range Two-step RT-PCR kit catalog no:205920 (Qiagen) according to the manufacturer's protocol. Amplification of carp TLR2ΔTIR (carp TLR2 sequence truncated at TIR domain) cDNA was performed as previously described (31). The vivid color™pcDNA™6.2/C-EmGFP-GW/TOPO® (Invitrogen) expression vector combined with TOPO®cloning was used to fluorescently label constructs by fusing TLR9 or TLR2ΔTIR to EmGFP at the C-terminal end. Isolation of highly pure plasmid DNA suitable for transfection was performed using S.N.A.P.™ Midi Prep Kit (Invitrogen, catalog no. K1910-01), according to the manufacturer´s protocol.

Macrophage cell cultures

Head kidney-derived macrophages, considered the fish equivalent of bone marrow-derived macrophages, were prepared as previously described (31)(32). Briefly, carp head kidneys were gently passed through a 100 µm sterile nylon mesh and cell suspensions were layered on 51% (1.07 $g.cm^{-3}$) Percoll. Macrophage cell cultures were initiated by seeding 1.75 x 10^7 head kidney leukocytes in a 75 cm^2 culture flask containing 20 ml of complete NMGFL-15 medium. Head kidney-derived macrophages, named macrophages throughout the manuscript, were harvested after 6 days of incubation at 27 °C by placing the flasks on ice for 10 min prior to gentle scraping.

Transient transfection of HEK 293 cells

Transient transfection of HEK 293 was performed as previously described (31). For transfection of HEK 293 cells 2 µg of TLR9-GFP or TLR2ΔTIR-GFP constructs were transfected by nucleoporation using nucleofactor™ solution V and program A-23 (Lonza Cologne AG, Germany) according to the manufacturer´s instructions. Forthy-eight hours post-transfection, cells were stimulated for 30 min with CpG ODN 1668, CpG ODN 2006, *E. coli* DNA or *T. borreli* DNA or left untreated as negative control. Cells were lysed for evaluation of phospho-p38 or phospho-tyrosine activity by Western blot.

Gene expression analysis

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) including the accompanying DNase I treatment on the columns, according to the manufacturer's protocol and stored at

-80°C until further use. Prior to cDNA synthesis, a second DN*ase* treatment was performed using DN*ase* I, Amplification Grade (Invitrogen). Synthesis of cDNA was performed with Invitrogen's SuperScript™ III First Strand Synthesis Systems for RT-PCR Systems using random primers according to the manufacturer's instructions. A non-reverse transcriptase control was included for each sample. cDNA samples were further diluted 50 times in nuclease-free water before use as template in real-time PCR experiments. Real time quantitative PCR (RT-qPCR) was performed in a 72-well Rotor-Gene™ 6000 (Corbett Research, Mortlake, Sydney, Australia) with the Brilliant® SYBR® Green QPCR (Stratagene, La Jolla, CA, USA) as detection chemistry as previously described (31). The primers used for RT-qPCR are listed in Table II. Fluorescence data from RT-qPCR experiments were analysed using Rotor-Gene version 6.0.21 software and exported to Microsoft Excel. The cycle threshold C_t for each sample and the reaction efficiencies (E) for each primer set were obtained upon Comparative Quantitation Analysis from the Rotor-Gene version 6.0.21 software. The relative expression ratio (R) of a target gene was calculated based on the E and the C_t deviation of sample versus control, and expressed relative to the S11 protein of the 40S subunit as reference gene.

Table II: Primers used for real-time quantitative PCR analysis

Primer	Sequence (5'-3')
IL-1β Fw	AAGGAGGCCAGTGGCTCTGT
IL-1β Fw	CCTGAAGAAGAGGAGGAGGCTGTCA
IFN-αβ Fw	GGCAGATATG+GGACGGTGAG
IFN-αβ Rv	GTCCTCCA+CCTCAGCTTTGTC
p35 Fw	TGCTTCTCTGTCTCTGTGATGGA
p35 Rv	CACAGCTGCAGTCGTTCTTGA
p40a Fw	GAGCGCATCAACCTGACCAT
p40a Rv	AGGATCGTGGATATGTGACCTCTAC

[a] The "+"is before the nucleic acid in which the locked nucleic acid bond was placed

Flow cytometric measurement of ROS production

Intracellular ROS levels were evaluated by FACScan® flow cytometer using the redox-sensitive dye dihydrorhodamine 123 (DHR) (33)(34) at 0.25 μg/ml (Sigma, D1054) as previously described. Macrophages were stimulated for 1 h with prototypical TLR9 ligands in the presence or absence of PMA. DHR was added to all samples, and cells incubated for 1 h at 27 °C. Cells were resuspended by pipetting, transferred to flow cytometer tubes and propidium iodide (PI; 0.1 mg/ml; Sigma) was added to each sample to detect and gate out PI^+ cells. For all cytometric measurements the same settings were used (FS 350 volt, gain 2; SS 700 volt, gain 10; FL1 600 volt; FL2 750 volt; FL3 675 volt; FL4 660 volt). The baseline offset was on and the FS discriminator was set at 50. Per sample, 10^4 events were measured by flow cytometer.

Nitrite production

Nitrite production was measured essentially as described before (35): to 75 µl of cell culture supernatant, 100 µl of 1% sulfanilamide in 2.5% (v/v) phosphoric acid and 100 µl of 0.1% (w/v) *N*-naphthyl-ethylenediamine in 2.5% (v/v) phosphoric acid were added in a 96-well flat-bottom plate. The absorbance was read at 540 nm (with 690 nm as a reference) and nitrite concentration (µM) was calculated by comparison with a sodium nitrite standard curve.

Western blot analysis

HEK 293 cells or carp macrophages were resuspended by pippeting and transferred to pre-cooled eppendorf tubes. Cells were washed twice in ice-cold PBS, lysed on ice with lysis solution [0.5% Triton X-100, 20 mM Tris, 100 mM NaCl, 1 mM EDTA, 50 mM NaF (Sigma), 5µM Na_3VO_4 (Sigma) 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma)], homogenized with a syringe and incubated for 10 min on ice. Cell lysates were centrifuged at 21000 *g* for 10 min at 4°C. Supernatant was collected and total protein content was determined by the Bradford method. Samples (20-25 µg) were boiled at 96 °C for 10 min with loading buffer containing β-mercaptoethanol and separated by 10% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes (Protrans, Schleicher & Schuell, Bioscience GmbH). Membranes were blocked in 5% w/v milk powder in TBS-T (10 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.5) for 1 h at room temperature and then incubated with primary antibody overnight at 4°C in 5% w/v BSA in TBS-T. The following antibodies were used: rabbit IgG anti-phospho-p38 (1:1000, Thr180/Tyr182, BioCat GmBh, Heidelberg, Germany), membranes were then incubated with goat-anti-mouse HRP-conjugated (1:1000, Dako, Glostrup, Denmark) or goat-anti-rabbit HRP-conjugated (1:2000, Dako) in 5% w/v milk powder in TBS-T for 1 h at room temperature. Between each incubation step, membranes were washed three times in TBS-T for 10 min at RT. Signal was detected by development with a chemoluminescence kit (Amersham) according to the manufacturer's protocol and visualized by the use of Lumni-fil chemiluminescent Detection Film (Roche, Woerden, The Netherlands).

Statistical Analysis

Transformed values (ln) were used for statistical analysis in SPSS software (version 17.0). Homogeneity of variance was analyzed using the Levene's test. Significant differences ($P \leq 0.05$) between a treatment (stimulated cells) and the control group (unstimulated cells) were determined by a one-way ANOVA followed by a Dunnett T-test. Significant differences between treatments ($P \leq 0.05$) were determined by one-way ANOVA followed by Bonferroni test. In case of unequal variances between treatments, the one-way ANOVA was followed by a Games–Howell test.

RESULTS

Activation of carp macrophages by prototypical TLR9 ligands

In mammalian vertebrates, TLR9–mediated activation of macrophages by DNA containing CpG motifs results in the secretion of cytokines such as IL-1β, IL-12 and type I interferons. A first series of experiments with CpG PS-ODN: 2216 (type-A), 1681 (type-B) and 2395 (type-C), in common use to stimulate Atlantic salmon leukocytes failed to show significant induction of cytokine (e.g.: IL-1β, IL-12) gene expression in carp macrophages (data not shown). Subsequently, CpG PS-ODN 1668 (type-B) and 2006 (type-B) were tested as these are commonly used to stimulate mouse or human cells, respectively, and have been proven effective cell stimulators in several fish species including seabream, rainbow trout and grass carp. These two CpG PS-ODNs of the B-type will be referred to as 'CpG ODN'. Bacterial *E. coli* DNA and protozoan parasite *T. borreli* DNA were also included for stimulation of carp macrophages. Optimal concentrations for CpG ODN (5 µM) and pathogen DNA (5 µg/ml) corresponded to optimal concentrations used for mammalian cells (data not shown). Both CpG ODNs and both sources of pathogen DNA modulated cytokine gene expression (IL-1β, p35, p40a and IFNαβ) in a time-dependent manner (Fig.1).

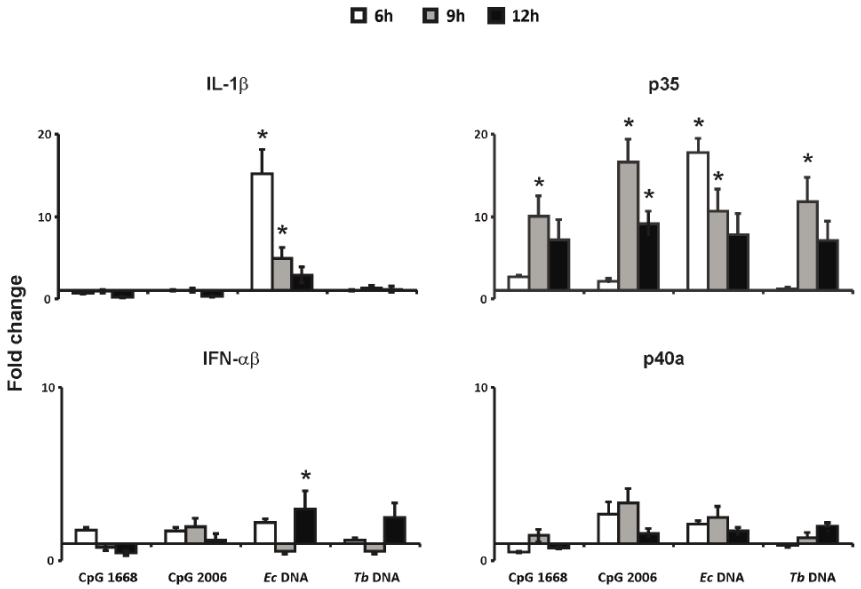


Figure 1. Kinetics of cytokine gene expression in carp macrophages after stimulation with different PAMPs. Real time-quantitative PCR analysis of gene expression in carp macrophages after stimulation for 6, 9 and 12h with CpG ODN1668 (class B; 5 µM), CpG ODN2006 (class B; 5 µM), *E. coli* DNA (5 µg/ml), *T. borreli* DNA (5 µg/ml) or left untreated as control. mRNA levels of IL-1β, IFNαβ, p35 and p40a relative to the house keeping 40S ribosomal protein gene level are expressed as fold change relative to unstimulated cells at time 0 h. Bars show averages ± SD of n=3 fish. *Significant ($p \leq 0.05$) difference compared with unstimulated cells. Note the differences in scale of the y-axes.

However, the magnitude and kinetics of the immune response were different between the stimulants. CpG ODNs as well as protozoan *T. borreli* DNA only significantly induced p35 gene expression. In contrast, bacterial *E. coli* DNA resulted in an early (6 h) and strong induction of IL-1β and p35 gene expression and to a lesser extent p40a and IFNαβ gene expression. In addition, the kinetics of carp p35 induction indicated highest gene expression at 6 h following *E. coli* DNA-stimulation, whereas CpG ODNs and *T. borreli* DNA stimulation induced highest p35 gene expression at 9 h (Fig. 1).

E. coli DNA induces phosphorylation-dependent cascades and NO production in carp macrophages

In mammalian vertebrates, recognition of PAMPs by TLR9 results in the activation of intracellular signaling pathways, mediated by protein kinases including IkB kinases and MAPKs, ultimately leading to cytokine secretion. Neither stimulation with the CpG ODNs, nor stimulation with protozoan *T. borreli* DNA resulted in MAPK-p38 activation of carp macrophages (Fig. 2A).

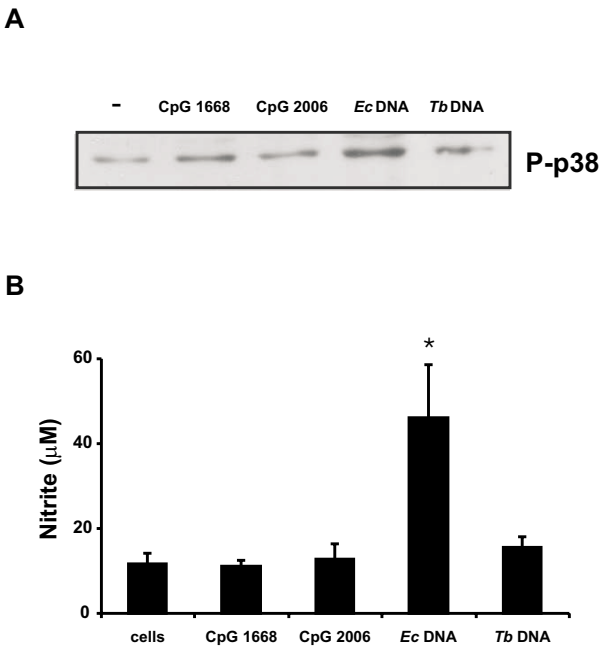


Figure 2. Effect of PAMPs on MAPK-p38 activation and NO production in carp macrophages. A. MAPK-p38 activation in carp macrophages analysed by immunoblotting for phospho-p38. Macrophages were stimulated with CpG ODN1668 (class B; 5 μM), CpG ODN2006 (class B; 5 μM), *E. coli* DNA (5 μg/ml), *T. borreli* DNA (5 μg/ml) for 30 min or left untreated as control. This is one experiment representative of n=3 experiments. B. NO production in carp macrophages. Macrophages were incubated with CpG ODN1668 (5 μM), CpG ODN2006 (5 μM), *E. coli* DNA (5 μg/ml), *T. borreli* DNA (5 μg/ml) for 18h or left untreated as control. Bars show average ± SD of n=4 fish. *Significant ($p \leq 0.05$) difference compared with unstimulated cells (control group).

In contrast, stimulation with *E. coli* DNA resulted in higher phosphorylation of MAPK-p38 in carp macrophages within 30 min (Fig. 2 A). In mammalian vertebrates, DNA with unmethylated CpG motifs stimulates leukocytes to generate reactive oxygen species (ROS, including O_2^- and NO· production). Neither CpG ODNs nor protozoan *T. borreli* DNA, at any concentration tested, stimulated ROS production measured by flow cytometric analysis of dihydrorhodamine 123 oxidation (data not shown). In contrast, *E. coli* DNA, but none of the other PAMPS tested, induced the production of nitric oxide in carp macrophages (Fig. 2B).

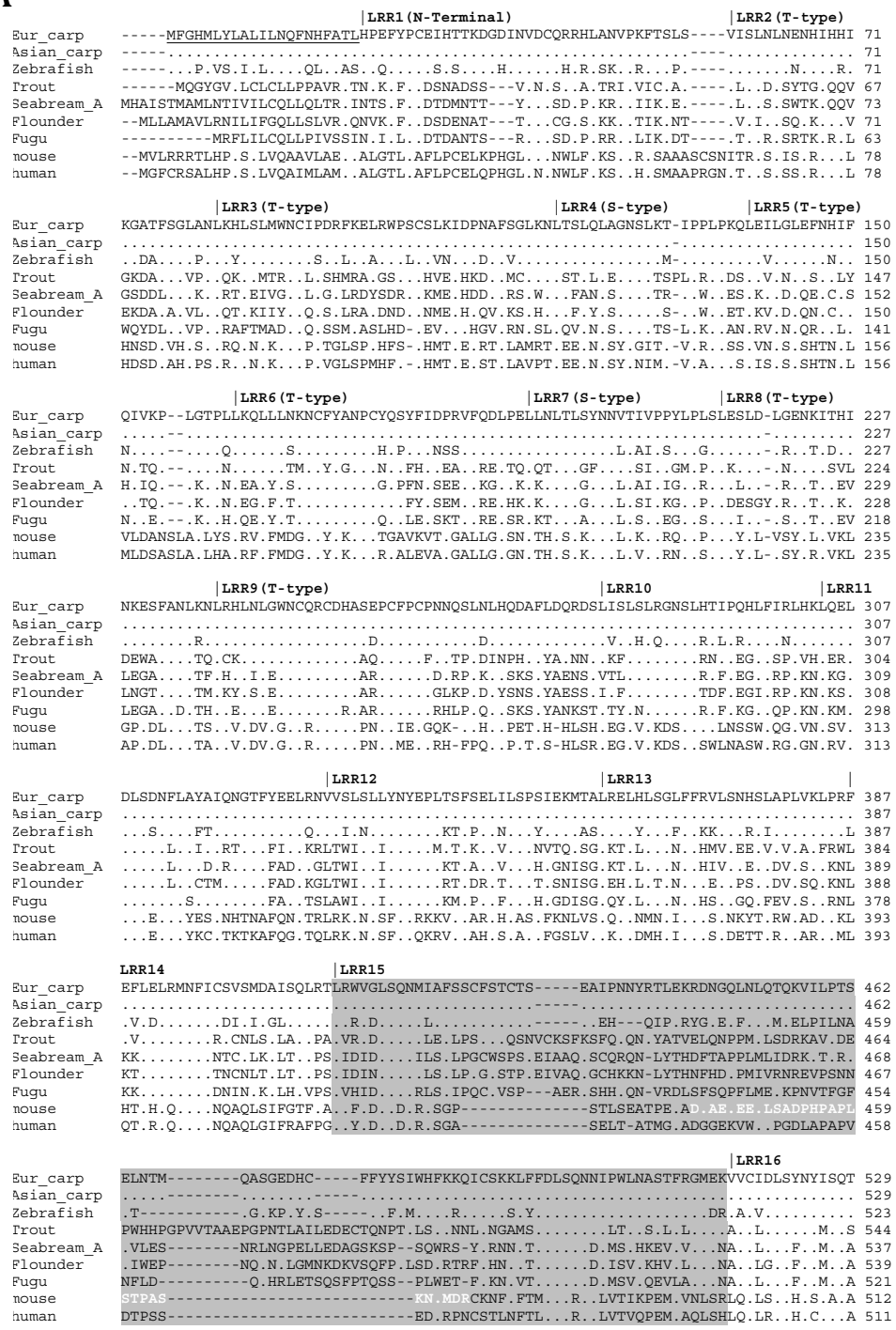
Carp TLR9 sequence analysis

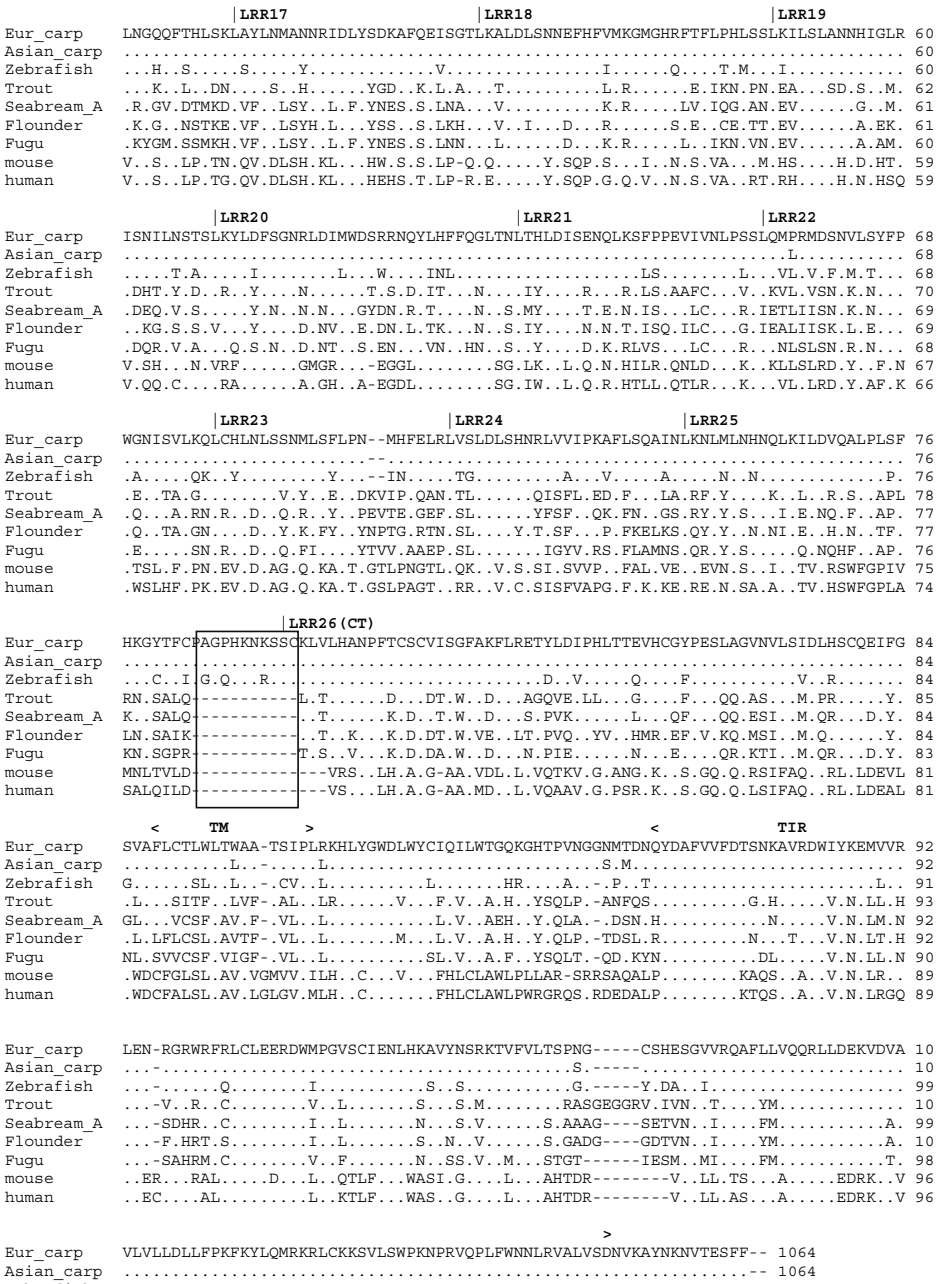
Using a homology cloning approach we obtained the full-length cDNA sequence coding for European common carp TLR9. Our mRNA sequence was 99% identical to the mRNA sequence for common carp TLR9 recently desposited in the database (Fig 3). The minor difference between these two sequences could possibly be due to a difference in template RNA owing to genetic differences between European and East-Asian subspecies of common carp (36). Our cDNA sequence of 3195bp codes for a TLR9 protein of 1064 amino acids (Fig. 3A). Sequence analysis confirmed conservation of structural features of TLRs including an extracellular leucine rich repeat (LRR) domain, a transmembrane domain and an intracellular TIR domain.

The extracellular domain of carp TLR9 has a predicted number of 26 LRRs (including the LRRs at the N-terminal and C-terminal region), similar to human and mouse TLR9 (Fig. 3A). Analysis of vertebrate LRR domains has defined two main types of LRR motifs: typical ("T", LxxLxLxxNxLxxLxxxxxF/LxxLxx) and bacterial ("S", LxxLxLxxNxLxxLPx(x)LPxx) motifs (37). In these motifs "L" can correspond to leucine (L), isoleucine (I), valine (V) or phenylalanine (F) and "N" can correspond to asparagine (N), threonine (T), Serine (S) or Cysteine (C). Vertebrate TLR9 has been classified to belong to the "TLR7 family" together with vertebrate TLR7 and TLR8. The N-terminal super-motif represented by "TT STT STT" is typical of the "TLR7 family" and is also present in carp TLR9. Region 780-789 in LRR25 seems present in cyprinid fish only.

In mouse, LRR15 (aa 441-470) comprises a flexible loop susceptible to proteolysis. Sequence analysis suggests the presence of an extended flexible loop in LRR15 of carp TLR9, and also in TLR9 of other teleost fish families. The amino acid sequence identity between the different fish species was particularly low for LRR15. This had a great effect on the degree of similarity between TLR9 sequences (Fig. 3B), despite the high similarity in number and distribution of LRR regions in the TLR9 ectodomain. The intracellular (TIR) domain showed a lower degree of variation among species, suggesting that downstream signalling cascades triggered by TLR9 activation may be conserved in carp.

A


|LRR1(N-Terminal)
|LRR2(T-type)
Eur_carp -----MFGHMLYLALILNQFNHFATLHPEFYPCEIHTTKDGDINVDCQRRHLANVPKFTSLS----VISLNLNENHIHHI 71
Asian_carp -----.....----..... 71
Zebrafish -------.P.VS.I.L....QL..AS..Q.....S.S....H......H.R.SK..R...P.----.....N....R. 71
Trout ------MQGYGV.LCLCLLPPAVR.TN.K.F..DSNADSS---V.N.S..A.TRI.VIC.A.----.L..D.SYTG.QQV 67
Seabream_A MHAISTMAMLNTIVILCQLLQLTR.INTS.F..DTDMNTT---Y...SD.P.KR..IIK.E.----.L..S.SWTK.QQV 73
Flounder --MLLAMAVLRNILIFGQLLSLVR.QNVK.F..DSDENAT---T...CG.S.KK..TIK.NT----.V.I..SQ.K...V 71
Fugu ----------MRFLILCQLLPIVSSIN.I.L..DTDANTS---R...SD.P.RR..LIK.DT----.T..R.SRTK.R.L 63
mouse --MVLRRRTLHP.S.LVQAAVLAE..ALGTL.AFLPCELKPHGL...NWLF.KS..R.SAAASCSNITR.S.IS.R...L 78
human --MGFCRSALHP.S.LVQAIMLAM..ALGTL.AFLPCELQPHGL.N.NWLF.KS..H.SMAAPRGN.T..S.SS.R...L 78
|LRR3(T-type)
|LRR4(S-type)
|LRR5(T-type)
Eur_carp KGATFSGLANLKHLSLMWNCIPDRFKELRWPSCSLKIDPNAFSGLKNLTSLQLAGNSLKT-IPPLPKQLEILGLEFNHIF 150
Asian_carp .....-..... 150
Zebrafish ..DA....P...Y........S..L..A...L..VN...D..V.............M-.........V......N.. 150
Trout GKDA...VP..QK..MTR..L.SHMRA.GS...HVE.HKD..MC....ST.L.E....TSPL.R..DS..V.N..S..LY 147
Seabream_A GSDDL...K..RT.EIVG..L.G.LRDYSDR..KME.HDD..RS.W...FAN.S....TR-..W..ES.K..D.QE.C.S 152
Flounder EKDA.A.VL..QT.KIIY..Q.S.LRA.DND..NME.H.QV.KS.H...F.Y.S.....S-..W..ET.KV.D.QN.C.. 150
Fugu WQYDL..VP..RAFTMAD..Q.SSM.ASLHD-.EV...HGV.RN.SL.QV.N.S....TS-L.K..AN.RV.N.QR..L. 141
mouse HNSD.VH.S..RQ.N.K...P.TGLSP.HFS-.HMT.E.RT.LAMRT.EE.N.SY.GIT.-V.R..SS.VN.S.SHTN.L 156
human HDSD.AH.PS.R..N.K...P.VGLSPMHF.-.HMT.E.ST.LAVPT.EE.N.SY.NIM.-V.A...S.IS.S.SHTN.L 156
|LRR6(T-type)
|LRR7(S-type)
|LRR8(T-type)
Eur_carp QIVKP--LGTPLLKQLLLNKNCFYANPCYQSYFIDPRVFQDLPELLNLTLSYNNVTIVPPYLPLSLESLD-LGENKITHI 227
Asian_carp .....--.....-..... 227
Zebrafish N....--....Q......S.........H.P...NSS.............L.AI.S...G......-.R..T.D.. 227
Trout N.TQ.--....N......TM..Y.G...N..FH..EA..RE.TQ.QT...GF....SI..GM.P..K...-.N....SVL 224
Seabream_A H.IQ.--.K..N.EA.Y.S.........G.PFN.SEE..KG..K.K....G...L.AI.IG..R...L..-.R..T..EV 229
Flounder ..TQ.--.K..N.EG.F.T.............FY.SEM..RE.HK.K....G...L.SI.KG..P..DESGY.R..T..K. 228
Fugu N..E.--.K..H.QE.Y.T.........Q..LE.SKT..RE.SR.KT...A...L.S..EG..S...I..-.S..T..EV 218
mouse VLDANSLA.LYS.RV.FMDG..Y.K...TGAVKVT.GALLG.SN.TH.S.K...L.K..RQ..P...Y.L-VSY.L.VKL 235
human MLDSASLA.LHA.RF.FMDG..Y.K...R.ALEVA.GALLG.GN.TH.S.K...L.V..RN..S...Y.L-.SY.R.VKL 235
|LRR9(T-type)
|LRR10
|LRR11
Eur_carp NKESFANLKNLRHLNLGWNCQRCDHASEPCFPCPNNQSLNLHQDAFLDQRDSLISLSLRGNSLHTIPQHLFIRLHKLQEL 307
Asian_carp 307
Zebrafish ........R.................D..........D.............V..H.Q....R.L.R....N..... 307
Trout DEWA....TQ.CK.............AQ.....F..TP.DINPH..YA.NN..KF........RN..EG..SP.VH.ER. 304
Seabream_A LEGA....TF.H..I.E.........AR......D.RP.K..SKS.YAENS.VTL.......R.F.EG..RP.KN.KG. 309
Flounder LNGT....TM.KY.S.E.........AR......GLKP.D.YSNS.YAESS.I.F........TDF.EGI.RP.KN.KS. 308
Fugu LEGA..D.TH..E...E.......R.AR......RHLP.Q..SKS.YANKST.TY.N......R.F.KG..QP.KN.KM. 298
mouse GP.DL...TS..V.DV.G..R.....PN..IE.GQK-..H..PET.H-HLSH.EG.V.KDS....LNSSW.QG.VN.SV. 313
human AP.DL...TA..V.DV.G..R.....PN..ME..RH-FPQ..P.T.S-HLSR.EG.V.KDS..SWLNASW.RG.GN.RV. 313
|LRR12
|LRR13
|
Eur_carp DLSDNFLAYAIQNGTFYEELRNVVSLSLLYNYEPLTSFSELILSPSIEKMTALRELHLSGLFFRVLSNHSLAPLVKLPRF 387
Asian_carp 387
Zebrafish ...S....FT..........Q...I.N........KT.P..N...Y....AS....Y...F..KK...R.I........L 387
Trout .....L..I..RT...FI..KRLTWI..I.....M.T.K..V...NVTQ.SG.KT.L...N..HMV.EE.V.V.A.FRWL 384
Seabream_A .....L...D.R....FAD..GLTWI..I......KT.A..V...H.GNISG.KT.L...N..HIV..E..DV.S..KNL 389
Flounder .....L..CTM.....FAD.KGLTWI..I......RT.DR.T...T.SNISG.EH.L.T.N...E..PS..DV.SQ.KNL 388
Fugu .......S........FA..TSLAWI..I......KM.P..F...H.GDISG.QY.L...N..HS..GQ.FEV.S..RNL 378
mouse ...E...YES.NHTNAFQN.TRLRK.N.SF..RKKV..AR.H.AS.FKNLVS.Q..NMN.I...S.NKYT.RW.AD..KL 393
human ...E...YKC.TKTKAFQG.TQLRK.N.SF..QKRV..AH.S.A..FGSLV..K..DMH.I...S.DETT.R..AR..ML 393
LRR14
|LRR15
Eur_carp EFLELRMNFICSVSMDAISQLRTLRWVGLSQNMIAFSSCFSTCTS-----EAIPNNYRTLEKRDNGQLNLQTQKVILPTS 462
Asian_carp .....-..... 462
Zebrafish .V.D.......DI.I.GL.....R.D.....L..........------..EH---QIP.RYG.E.F...M.ELPILNA 459
Trout .V........R.CNLS.LA..PA.VR.D.....LE.LPS...QSNVCKSFKSFQ.QN.YATVELQNPPM.LSDRKAV.DE 464
Seabream_A KK........NTC.LK.LT..PS.IDID....ILS.LPGCWSPS.EIAAQ.SCQRQN-LYTHDFTAPPLMLIDRK.T.R. 468
Flounder KT........TNCNLT.LT..PS.IDIN.....LS.LP.G.STP.EIVAQ.GCHKKN-LYTHNFHD.PMIVRNREVPSNN 467
Fugu KK........DNIN.K.LH.VPS.VHID....RLS.IPQC.VSP---AER.SHH.QN-VRDLSFSQPFLME.KPNVTFGF 454
mouse HT.H.Q....NQAQLSIFGTF.A..F.D..D.R.SGP--------------STLSEATPE.AD.AE.EE.LSADPHPAPL 459
human QT.R.Q....NQAQLGIFRAFPG..Y.D..D.R.SGA--------------SELT-ATMG.ADGGEKVW..PGDLAPAPV 458
|LRR16
Eur_carp ELNTM--------QASGEDHC-----FFYYSIWHFKKQICSKKLFFDLSQNNIPWLNASTFRGMEKVVCIDLSYNYISQT 529
Asian_carp ....---------.....-----..... 529
Zebrafish .T-----------.G.KP.Y.S------..F.M....R.....S.Y.................DR.A.V..... 523
Trout PWHHPGPVVTAAEPGPNTLAILEDECTQNPT.LS..NNL.NGAMS........LT..S.L.L....A..L.....M..S 544
Seabream_A .VLES--------NRLNGPELLEDAGSKSP--SQWRS-Y.RNN.T......D.MS.HKEV.V...NA..L...F..M..A 537
Flounder .IWEP--------NQ.N.LGMNKDKVSQFP.LSD.RTRF.HN..T......D.ISV.KHV.L...NA..LG..F..M..A 539
Fugu NFLD----------Q.HRLETSQSFPTQSS--PLWET-F.KN.VT......D.MSV.QEVLA...NA..L...F..M..A 521
mouse STPAS-------------------------KN.MDRCKNF.FTM...R..LVTIKPEM.VNLSRLQ.LS..H.S.A.A 512
human DTPSS-------------------------ED.RPNCSTLNFTL...R..LVTVQPEM.AQLSHLQ.LR..H.C...A 511


LRR17
LRR18
LRR19
Eur_carp LNGQQFTHLSKLAYLNMANNRIDLYSDKAFQEISGTLKALDLSNNEFHFVMKGMGHRFTFLPHLSSLKILSLANNHIGLR 60
Asian_carp 60
Zebrafish ...H..S.....S.....Y.....V.....I.....Q....T.M...I..... 60
Trout ...K..L..DN....S..H.....YGD..K.L.A...T.....L.R.....E.IKN.PN.EA...SD.S..M. 62
Seabream_A .R.GV.DTMKD.VF..LSY..L.F.YNES.S.LNA...V.....K.R.....LV.IQG.AN.EV.....G..M. 61
Flounder .K.G..NSTKE.VF..LSYH.L...YSS..S.LKH...V..I...D...R.....S.E..CE.TT.EV.....A.EK. 61
Fugu .KYGM.SSMKH.VF..LSY..L.F.YNES.S.LNN...L.....D...K.R.....L..IKN.VN.EV.....A.AM. 60
mouse V..S..LP.TN.QV.DLSH.KL...HW.S.S.LP-Q.Q.....Y.SQP.S...I..N.S.VA...M.HS....H.D.HT. 59
human V..S..LP.TG.QV.DLSH.KL...HEHS.T.LP-R.E.....Y.SQP.G.Q.V..N.S.VA..RT.RH....H.N.HSQ 59
LRR20
LRR21
LRR22
Eur_carp ISNILNSTSLKYLDFSGNRLDIMWDSRRNQYLHFFQGLTNLTHLDISENQLKSFPPEVIVNLPSSLQMPRMDSNVLSYFP 68
Asian_carp .....L..... 68
Zebrafish .....T.A.....I.....L...W....INL.....LS.....L...VL.V.F.M.T... 68
Trout .DHT.Y.D..R..Y....N.....T.S.D.IT...N....IY....R...R.LS.AAFC...V..KVL.VSN.K.N... 70
Seabream_A .DEQ.V.S.....Y.N..N.N...GYDN.R.T....N..S.MY....T.E.N.IS...LC...R.IETLIISN.K.N... 69
Flounder ..KG.S.S.V...Y....D.NV..E.DN.L.TK...N..S.IY....N.N.T.ISQ.ILC...G.IEALIISK.L.E... 69
Fugu .DQR.V.A...Q.S.N..D.NT..S.EN...VN..HN..S..Y....D.K.RLVS...LC...R...NLSLSN.R.N... 68
mouse V.SH...N.VRF.....GMGR...-EGGL.....SG.LK..L.Q.N.HILR.QNLD...K..KLLSLRD.Y..F.N 67
human V.QQ.C....RA.....A.GH..A-EGDL.....SG.IW..L.Q.R.HTLL.QTLR...K...VL.LRD.Y.AF.K 66
LRR23
LRR24
LRR25
Eur_carp WGNISVLKQLCHLNLSSNMLSFLPN--MHFELRLVSLDLSHNRLVVIPKAFLSQAINLKNLMLNHNQLKILDVQALPLSF 76
Asian_carp .....--..... 76
Zebrafish .A.....QK..Y.....Y...--IN.....TG.....A...V.....A.....N..N.....P. 76
Trout .E..TA.G.....V.Y..E..DKVIP.QAN.TL.....QISFL.ED.F...LA.RF.Y....K..L..R.S..APL 78
Seabream_A .Q...A.RN.R..D..Q.R..Y..PEVTE.GEF.SL.....YFSF..QK.FN..GS.RY.Y.S...I.E.NQ.F..AP. 77
Flounder .Q..TA.GN....D..Y.K.FY..YNPTG.RTN.SL....Y.T.SF...P.FKELKS.QY.Y..N.NI.E..H.N..TF. 77
Fugu .E.....SN.R..D..Q.FI....YTVV.AAEP.SL.....IGYV.RS.FLAMNS.QR.Y.S.....Q.NQHF..AP. 76
mouse .TSL.F.PN.EV.D.AG.Q.KA.T.GTLPNGTL.QK..V.S.SI.SVVP..FAL.VE..EVN.S..I..TV.RSWFGPIV 75
human .WSLHF.PK.EV.D.AG.Q.KA.T.GSLPAGT..RR..V.C.SISFVAPG.F.K.KE.RE.N.SA.A..TV.HSWFGPLA 74
LRR26(CT)
Eur_carp HKGYTFCPAGPHKNKSSCKLVLHANPFTCSCVISGFAKFLRETYLDIPHLTTEVHCGYPESLAGVNVLSIDLHSCQEIFG 84
Asian_carp 84
Zebrafish ...C..I.G.Q...R.....D..V.....Q....F.....V..R..... 84
Trout RN.SALQ----------L.T.....D...DT.W..D...AGQVE.LL...G....F...QQ.AS...M.PR.....Y. 85
Seabream_A K..SALQ----------..T.....K.D..T.W..D...S.PVK.....L...QF...QQ.ESI..M.QR...D.Y. 84
Flounder LN.SAIK----------..T..K...K.D.DT.W.VE..LT.PVQ..YV..HMR.EF.V.KQ.MSI..M.Q.....Y. 84
Fugu KN.SGPR----------T.S..V...K.D.DA.W..D...N.PIE.....N...E....QR.KTI..M.QR...D.Y. 83
mouse MNLTVLD----------VRS..LH.A.G-AA.VDL.L.VQTKV.G.ANG.K..S.GQ.Q.RSIFAQ..RL.LDEVL 81
human SALQILD----------VS...LH.A.G-AA.MD..L.VQAAV.G.PSR.K..S.GQ.Q.LSIFAQ..RL.LDEAL 81
TM
TIR
Eur_carp SVAFLCTLWLTWAA-TSIPLRKHLYGWDLWYCIQILWTGQKGHTPVNGGNMTDNQYDAFVVFDTSNKAVRDWIYKEMVVR 92
Asian_carp .....L..-.....L.....S.M..... 92
Zebrafish G.....SL..L..-.CV..L.....L.....HR....A..-.P..T.....L.. 91
Trout .L...SITF..LVF-.AL..LR.....V...F.V..A.H..YSQLP.-ANFQS.....G.H.....V.N.LL.H 93
Seabream_A GL...VCSF.AV.F-.VL..L.....L.V..AEH..Y.QLA.-.DSN.H.....N.....V.N.LM.N 92
Flounder .L.LFLCSL.AVTF-.VL..L.....M...L.V..A.H..Y.QLP.-TDSL.R.....N...T...V.N.LT.H 92
Fugu NL.SVVCSF.VIGF-.VL..L.....SL.V..A.F..YSQLT.-QD.KYN.....DL.....V.N.LL.N 90
mouse .WDCFGLSL.AV.VGMVV.ILH..C...V...FHLCLAWLPLLAR-SRRSAQALP.....KAQS..A..V.N.LR.. 89
human .WDCFALSL.AV.LGLGV.MLH..C.....FHLCLAWLPWRGRQS.RDEDALP.....KTQS..A..V.N.LRGQ 89
Eur_carp LEN-RGRWRFRLCLEERDWMPGVSCIENLHKAVYNSRKTVFVLTSPNG-----CSHESGVVRQAFLLVQQRLLDEKVDVA 10
Asian_carp ...-.....S.....-----..... 10
Zebrafish ...-.....Q.....I.....S..S.....G.-----Y.DA..I..... 99
Trout ...-V..R..C.....V..L.....S...S.M.....RASGEGGRV.IVN..T....YM..... 10
Seabream_A ...-SDHR..C.....I..L.....N...S.V.....S.AAAG----SETVN..I....FM.....A. 99
Flounder ...-F.HRT.S.....I..L.....S..N..V.....S.GADG----GDTVN..I....YM.....A. 10
Fugu ...-SAHRM.C.....V..F.....N..SS.V..M...STGT------IESM..MI....FM.....T. 98
mouse ..ER...RAL.....D...L..QTLF...WASI.G....L...AHTDR--------V..LL.TS...A.....EDRK..V 96
human ..EC....AL.....L..KTLF...WAS..G....L...AHTDR--------V..LL.AS...A.....EDRK..V 96
Eur_carp VLVLLDLLFPKFKYLQMRKRLCKKSVLSWPKNPRVQPLFWNNLRVALVSDNVKAYNKNVTESFF-- 1064
Asian_carp .....-- 1064
Zebrafish .....F.....R.....D.....R.....-- 1057
Trout .....EV...L....L.....R.F.....R..QA.....HMKT..A..SIRS.DS..N...I-- 1074
Seabream_A .....EM...L....L.R...R.....R...A.....RV.M..S...L.F.DN.MS...M-- 1063
Flounder .....EM...L....L.....R....T.....KA.....E..M..S...L.L.DN.MS...V-- 1065
Fugu .....EM...L....L.....R.....R..KA.....Q..M..S...LSF.DN.MS...I-- 1045
mouse ...I.RPDAHRSR.VRL.Q...RQ...F..QQ.NG.GG..AQ.ST..TR..RHF..Q.FCRGPTAE 1032
human ...I.SPDGRRSR.VRL.Q...RQ...L..HQ.SG.RS..AQ.GM..TR..HHF..R.FCQGPTAE 1032

B

		TELEOST FISH						MAMMALS	
		Cypriniformes		*Salmoniformes*	*Perciformes*	*Pleuronectiformes*	*Tetraodontiformes*	*Rodentia*	*Primates*
		ASIAN CARP	ZEBRAFISH	TROUT	SEABREAM	FLOUNDER	FUGU	MOUSE	HUMAN
EUROPEAN CARP	LRR	99	78	49	46	45	46	36	36
	TIR	99	91	74	72	72	70	52	53

Figure 3. Alignment of carp TLR9 with fish and mammalian vertebrate TLR9 proteins. A. European common carp TLR9 and East-Asian common carp are indicated as Eur_carp and Asian_carp, respectively. Dots indicate identities to the carp TLR9 sequence and dashes denote gaps used to maximize the alignment. Leucine-rich repeats (LRR), transmembrane and Toll/IL-1 Receptor (TIR) domains predicted for European common carp are shown in bold above the sequence. Symbol (|) indicates the first putative amino acid residue of each LRR motif. The predicted signal peptide is underlined. Putative mouse TLR9 cleavage region encompassing 441-470 residues is shown in (white) bold localized in LRR15 (grey shaded). The region encompassing 780-789 residues in LRR25, present in cypriniformes only, is boxed. Transmebrane (TM) region and Toll/IL-1Receptor (TIR) domain are indicated above the sequence. B. Percentage sequence identity for LRR and TIR domains between carp, other fish and mammalian vertebrate TLR9 proteins, determined by Clustal W alignment. GenBank accession nos: East-Asian common carp (*Cyprinus carpio haemotopterus,* ADE20130.1)[1], zebrafish (*Danio rerio,* NP_001124066.1), rainbow trout (*Onchorhynchus mykiss,* NP_001123463.1), gilthead seabream A (*Sparus aurata*, AAW81697.1), Japanase flounder (*Paralichthys olivaceus,* BAE80691.1), pufferfish (Takifugu rubripes, AAW69377.1), mouse (*Mus musculs,* NP_112455.1) and human (*Homo sapiens*, NP_059138.1). [1] This sequence was reported as common carp TLR9 (11). It is unclear from the publication if the template originated from European common carp, from East-Asian ('koi') common carp, or from both. Given the difference between our TLR9 sequence and the reported sequence, we suggest the reported TLR9 sequence could be from the East-Asian subspecies of common carp.

E .coli DNA is a ligand of carp TLR9

HEK 293 cells were transfected with full-length carp TLR9 (TLR9 WT), or with truncated Toll-like receptor TLR2 (TLR2ΔTIR) as negative control, and phosphorylation of MAPK-p38 was used as a measure for responsiveness to different TLR9 ligands. Stimulation of TLR2ΔTIR transfected HEK 293 cells with prototypical TLR9 ligands did not increase MAPK-p38 phosphorylation (Control; Fig. 4). This negative control thereby showed the unresponsiveness of the parental HEK 293 cells to these TLR9 agonists, confirming that HEK 293 cells themselves do not bear receptors for any of the PAMPs analysed. In contrast, stimulation with *E. coli* DNA, but not stimulation with *T. borreli* DNA nor with CpG ODN resulted in an increase of MAPK p38 activity (TLR9; Fig. 4). Our results suggest that carp TLR9 can recognize *E. coli* DNA.

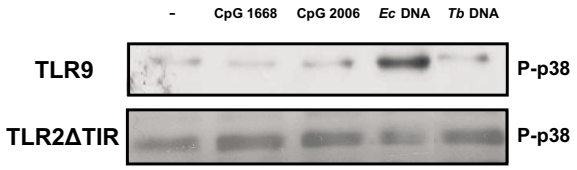


Figure 4. Activation of carp TLR9 by different PAMPs in HEK 293 cells. TLR9 and control (TLR2ΔTIR) transfected HEK293 cells were stimulated with CpG ODN1668 (class B; 5 μM), CpG ODN2006 (class B; 5 μM), *E. coli* DNA (5 μg/ml), T. borreli DNA (5 μg/ml) for 30 min or left untreated as control. MAPK-p38 activation in human HEK 293 cells analysed by immunoblotting for phospho-p38. This is one experiment representative of three experiments.

Activation of carp macrophages by the TLR9 ligand E. coli DNA is protease dependent

In mammalian vertebrates, full-length TLR9 is cleaved by resident proteases in de endolysosome to generate a truncated, functional TLR9 molecule. In mouse, LRR15 of TLR9 comprises a flexible loop that can render the molecule susceptible to proteolysis. Sequence analysis suggested the presence of an extended flexible loop in LRR15 of carp TLR9. To scrutinize the structure of this extended flexible loop, we modelled the ectodomain of carp TLR9 on the crystal structure of mouse TLR3. The flexible loop in 'fish' TLR9 is much longer than the one in mouse TLR9 (see Fig. 3A). Therefore, no 'template' for reliable modelling was available for this particular region. However, the presence of an extended flexible loop in LRR15 of carp TLR9, in a similar position as the flexible loop of mouse TLR9, is apparent (Fig. 5A).

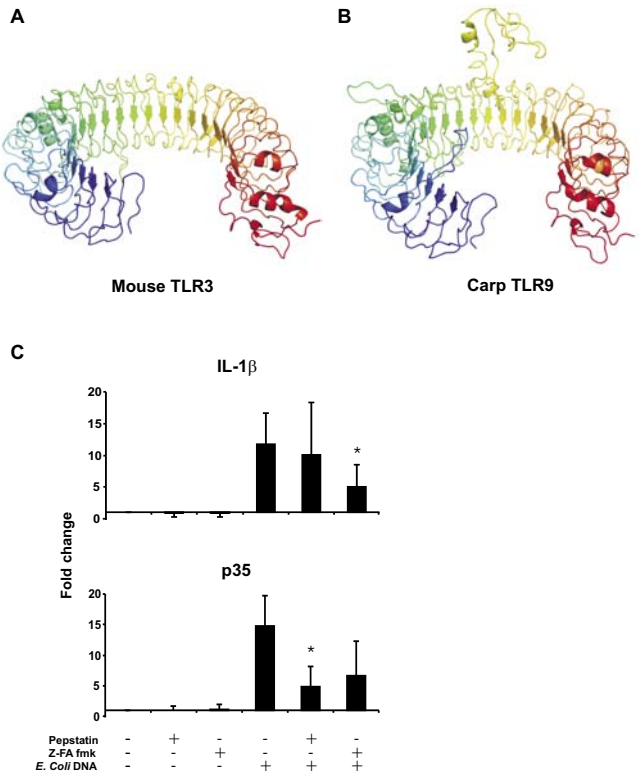


Figure 5. Analysis of carp TLR9 susceptibility for protease activity. A. Three-dimensional model for mouse TLR9 based on the crystal structure of mouse TLR3. B. Three-dimensional model for carp TLR9 based on the crystal structure of mouse TLR3. The N-terminal LRR is denoted in blue and the C-terminal LRR in red. Note the presence of an extended flexible loop for carp TLR9 in the middle region of the molecule (LRR15, yellow). C. Real time-qPCR analysis of gene expression in carp macrophages after stimulation for 6, with *E. coli* DNA (5 μg/ml) in the presence of inhibitor of cysteine protease (Z-FAD-fmk, 10 μM) or inhibitor of aspartic proteases (pepstatin A, 10 μM) or left untreated as control. mRNA levels of IL-1β and p35 relative to the house keeping 40S ribosomal protein gene level are expressed as relative to unstimulated cells. Bars show average ± SD of n=4 fish. *Significant ($p \leq 0.05$) difference compared with *E. coli* DNA-stimulated cells.

The presence of a flexible loop in carp TLR9 suggested a role for proteases in the regulation of carp TLR9 activation. To test this hypothesis we stimulated carp macrophages with the TLR9 ligand *E. coli* DNA and applied to these cells inhibitors of cysteine proteases and of aspartic proteases. Z-FA-fmk (inhibitor of cysteine proteases) and Pepstatin A (inhibitor of aspartic proteases) reduced IL-1β and p35 cytokine gene expression, respectively, in *E. coli*-stimulated carp macrophages (Fig. 5B). Our results suggest that activation of carp TLR9 can be a process susceptible to proteolysis.

DISCUSSION

The ability to differentiate between pathogenic and host DNA represents an important element of the eukaryotic immune system to protect against infection while avoiding inappropriate and pathologic immune responses to self-DNA. In mammalian vertebrates, ligand specificity of TLR9 for synthethic PS-modified ODNs with unmethylated CpG motifs has been confirmed in cells from TLR9-deficient mice (38) and in cells transfected with human or mouse TLR9 genes (39). We studied as putative ligands for carp TLR9 several CpG PS-ODNs, selected on the basis of studies in other (fish) species, but also protozoan parasite *T. borreli* DNA and single-sheared *Escherichia coli* DNA complexed with the lipid-based transfection reagent LyoVec. Until present, for no fish species had a clear proof been published linking prototypical TLR9 ligands to the presence of a TLR9 protein. Transfection of HEK 293 cells with carp TLR9 established the ability of carp TLR9 to recognize bacterial *E. coli* DNA, but not the selected CpG PS-ODNs nor protozoan parasite *T. borreli* DNA.

Distinguishing pathogen DNA from host DNA has been proposed a multifactorial process based on three main factors: (i) suppression of CpG dinucleotide frequency and enhanced cytosine methylation in the CpG motifs in the host DNA (5)(40), (ii) differences in CpG sequence motifs (41) (42) and (iii) mobilization of TLR9 to the DNA-containing endosome (43)(19).

(i) Mammalian vertebrate DNA has a significant lower frequency of unmethylated CpG motifs than viral or bacterial DNA, of which 70-80% CpG are methylated (3). Interestingly, aquatic vertebrate (fish, amphibians) DNA has an average methylation value approximately twice as high as reported for birds, reptiles and mammals (44)(45)(46). This implies that fish TLR9 receptors certainly should be able to distinguish pathogen DNA from host DNA based on differences in CpG dinucleotide frequency and methylation status.

(ii) Stimulatory CpG sequence motifs have been reported species-specific (41). The GTCGTT motif seems optimal for human TLR9 (47) whereas the GACGTT motif has the greatest activity in mice (48). We initially studied the stimulatory effect of CpG motifs known to stimulate Atlantic salmon leukocytes (15) but could not detect stimulating effects on carp macrophages, confirming species specificity of CpG sequence motifs within teleost fishes. Subsequent study of CpG motifs chosen on the basis of studies in mouse and human and known to stimulate leukocytes from gilthead seabream, rainbow trout and grass carp (ODN 1668 and 2006) could stimulate carp macrophages to some extent (p35 gene expression)

but could not clearly stimulate HEK 293 cells transfected with carp TLR9. Although differences in immunostimulating capacity of CpG ODNs can also be due to differences in target cells or differences in uptake, our findings suggest a (fish) species-dependent CpG binding specificity.

We used bacterial *E. coli* DNA complexed with a lipid-based transfection reagent. Stimulation of carp macrophages with this complexed bacterial DNA resulted in much stronger cellular activation as shown by cytokine gene expression, nitrogen radical production and MAPK-p38 activation when compared to uncomplexed *E. coli* DNA (unpublished data), CpG ODNs or trypanosomal DNA. It has been previously shown that DNA from the protozoan parasite *T. borreli*, although at high concentrations of 50 μg/ml, induced nitric oxide (NO) production in carp head-kidney phagocytes. DNAse-treatment and CpG methylation treatment lowered the production of NO, suggesting that CpG motifs in *T. borreli* DNA could stimulate carp phagocytes, possibly via activation of TLR9 (24). We could not confirm that *T. borreli* DNA is a ligand of carp TLR9 at physiological concentrations. In general, (eukaryotic) trypanosome DNA has been recognised a weaker inducer of cell activation than bacterial DNA. This can be explained, in part, by the unmethylated CpG frequency of nuclear trypanosome DNA that is lower than that of bacterial DNA, but still greater than that found in DNA of vertebrates (49). In addition, the kinetoplast, which is the second DNA-containing organelle in trypanosomes, suppresses the CpG frequency in trypanosome DNA (50). It cannot be excluded, however, that complexed trypanosome DNA, alike the ssDNA/LyoVec *E. coli* used in this study, would be more efficiently recognized by carp TLR9.

(iii) Mobilization of TLR9 to the endosome has not been studied in fish. In mice, differences in immunostimulating capacity of CpG ODNs are primarily determined by the ability to induce mobilization of TLR9 and colocalization with the TLR9 receptor in the endosomal compartment. Liposomal nanoparticles, encapsulating PS CpG ODN, endowed immunostimulating capacity of both unmethylated and methylated CpG ODN through a TLR9-mediated mechanism, confirming an important role for colocalization (51). TLR9 activation is achieved after cleavage in the endosome by resident proteases and the truncated from of TLR9 is present exclusively in endosomes. The flexible loop present in the ectodomain of TLR9 is the target region for this protease activity (21)(22). Homology modelling of the ectodomain of carp TLR9, as well as the ectodomain of several other fish species, confirmed the presence of an extended flexible loop in TLR9 of fishes. However, the functional consequence of an extended length of the flexible loop region for the cleavage of fish TLR9 by host proteases remains uninvestigated. Our experiments did show that the presumed TLR9-dependent activation of carp macrophages by *E. coli* DNA was dependent on the presence of protease activity. Although the exact proteases responsible for this process have not yet been identified, cysteine proteases appear to play an important role.

Our results suggest an evolutionary conservation of the regulation of carp TLR9 activation by host proteases. Future experiments including overexpression of TLR9 in carp macrophages followed by inhibition of protease activity and detection of the truncated

form of TLR9 by western blot will help elucidate the proteolytic regulation of carp TLR9.

ACKNOWLEDGMENTS

We thank the central facilities, "De Haar-Vissen", for taking care of the carp. Trudi Hermsen, Anja J. Taverne-Thiele and Danilo Pietretti are gratefully acknowledged for technical support.

FUNDING

This work was supported by the Portuguese foundation Fundação para a Ciência e Tecnologia under Grant agreement number SFR/BD/21522/2005.

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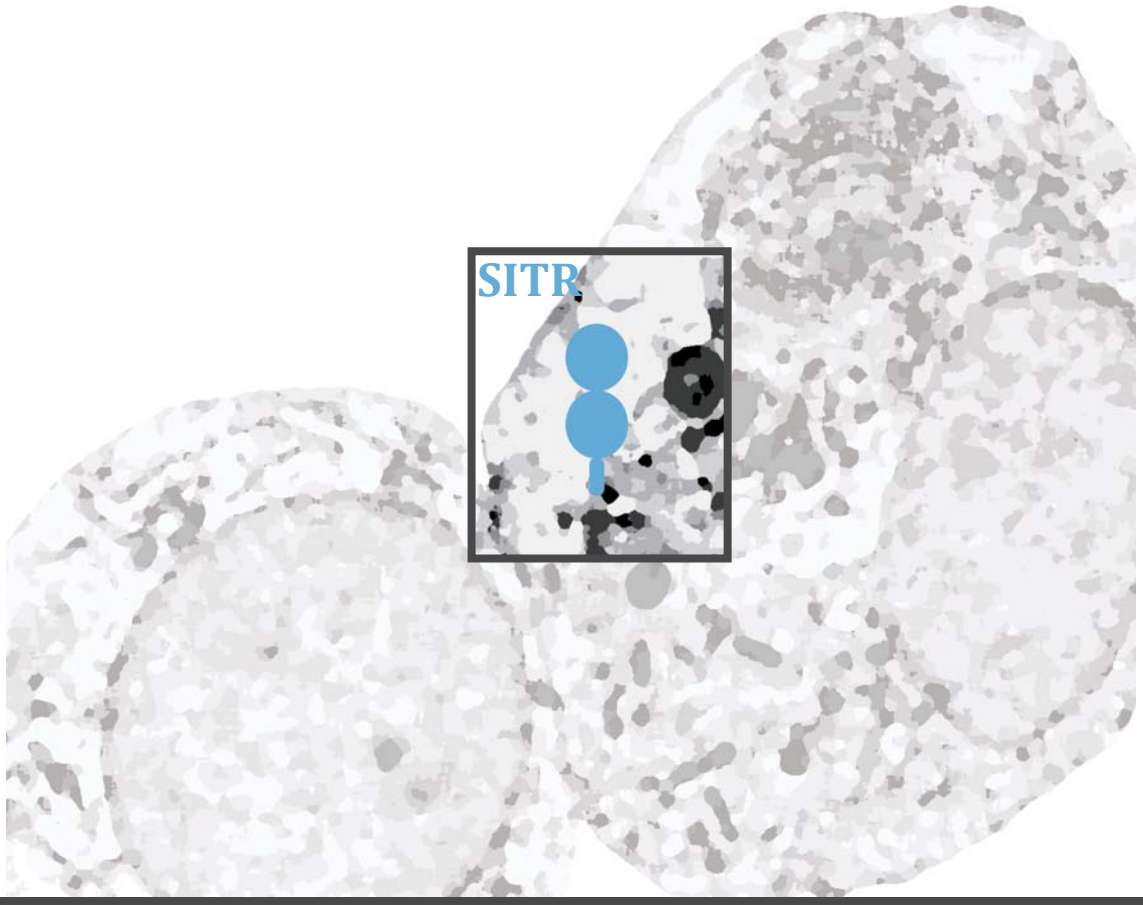
" You only know if a parachute works
if you dare to jump. "

Loesje



Chapter 7

A novel Soluble Immune-Type Receptor (SITR) in teleost fish: Carp SITR is involved in the NO-mediated response to a protozoan parasite

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Submitted manuscript

ABSTRACT

The innate immune system relies upon a wide range of germ-line encoded receptors including a large number of immunoglobulin superfamily (IgSF) receptors. Different Ig-like immune receptor families have been reported in mammals, birds, amphibians and fish. Most innate immune receptors of the IgSF are type I transmembrane proteins containing one or more extracellular Ig-like domains and their regulation of effector functions is mediated intracellularly by distinct stimulatory or inhibitory pathways. Carp SITR was found in a subtracted cDNA repertoire from carp macrophages, enriched for genes up-regulated in response to the protozoan parasite *Trypanoplasma borreli*. Carp SITR is a type I protein with two extracellular Ig domains in a unique organisation of a N-proximal V/C2 (or I-) type and a C-proximal V-type Ig domain, devoid of a transmembrane domain or any intracytoplasmic signalling motif. The carp SITR C-proximal V-type Ig domain, in particular, has a close sequence similarity and conserved structural characteristics to the mammalian CD300 molecules. By generating an anti-SITR antibody we could show that SITR protein expression was restricted to cells of the myeloid lineage. Carp SITR is abundantly expressed in macrophages and secreted upon in vitro stimulation with the protozoan parasite *T. borreli*. Secretion of SITR protein during *in vivo T. borreli* infection suggests a role for this IgSF receptor in the host response to this protozoan parasite. Overexpression of carp SITR in mouse macrophages and knock-down of SITR protein expression in carp macrophages provided evidence for the involvement of carp SITR in the parasite-induced NO production. We report the structural and functional characterization of a novel soluble immune-type receptor (SITR) in a teleost fish and propose a role for carp SITR in the NO-mediated response to a protozoan parasite.

INTRODUCTION

The innate immune system is an ancient form of host defense that relies upon a wide range of non-rearranging, germ-line encoded receptors including a large number of immunoglobulin superfamily (IgSF) receptors (1, 2). Members of the IgSF typically contain at least one Ig domain of about 100 amino acids built up by a sandwich of two ß-sheets of antiparallel ß-strands packed together and roughly forming a barrel-shaped structure. Ig domains are either of the variable (V) type, the constant (C)1 or C2 types or the intermediate (I) type differing by varying numbers of ß-strands in each of the ß-sheets that form the sandwich (3, 4). The number and organisation of the Ig domains in surface bound proteins of the IgSF may vary, but usually the N-terminal Ig domain is of the V-type, whereas the remaining domain(s) are of the C1 or C2-type (5, 6).

Well-studied Ig-like immune receptors comprise the leukocyte receptor cluster (LRC) on human chromosome 19 (7). LRC genes can be grouped into different multigene families, which induce leukocyte-Ig-like receptors (LILRs), Ig-like transcripts (ILTs) (8), killer inhibitory receptors (KIRs) (9), platelet collagen receptor glycoprotein VI (GPVI) (10), receptor for IgAFc (FCAR) (11), natural cytotoxicity receptor (NCR) NKp46 (12) and leukocyte-associated inhibitory receptors (LAIRs) (13). In addition to the LRC, two other small clusters have been identified in the human genome on chromosome 6 and 17. A cluster of single V-type Ig domain innate immune receptors on human chromosome 6 includes the natural cytoxicity receptor NKp44, triggering receptors expressed on myeloid cells (TREM) and TREM-like transcripts (TLT) (14, 15). More distant relatives of TREM proteins are the CD300 family found on human chromosome 17, as well as the polymeric Ig receptor (pIgR). CD300 molecules are glycoproteins with a single V-type Ig domain with a conserved YWCR amino acid motif and two (instead of one) disulfide bonds (16-18). Most innate immune receptors of the IgSF are type I transmembrane proteins containing one or more extracellular Ig-like domains, a transmembrane segment and a cytoplasmic region that may contain tyrosine residues (2). Typically, their regulation of effector function is mediated intracellularly by distinct stimulatory or inhibitory pathways. Stimulatory receptors have a short cytoplasmic tail devoid of canonical signalling motifs but contain a positively charged amino acid residue within their transmembrane region that allows the receptor to associate with ITAM (immune receptor tyrosine-based activation motifs)-containing transmembrane adaptor proteins (19, 20). Inhibitory receptors have long cytoplasmic tails with a variable number of ITIMs (immune receptor tyrosine-based inhibition motifs) (21, 22).

Soluble receptors can be generated by several mechanisms, which include proteolytic cleavage of receptor ectodomains, alternative splicing of mRNA transcripts or transcription of distinct genes that encode soluble receptors (23). In mammals, LILRA3 and LAIR2 encoded within the LRC, are devoid of a transmembrane region and are secreted rather than embedded in the cell membrane (24, 25). In addition, soluble forms of the TREM family members (TREM-1, TREM-2 and TLT-1) have been described (26-28). Although their function is unknown, sTREM-1 and sTREM-2 are thought to negatively regulate TREM receptors signaling through neutralization of the respective ligands. Some reports suggest that soluble TREM are obtained by alternative splicing of mRNA transcripts whereas others report origination by proteolytic cleavage of the receptors' ectodomain (29).

Studies in non-mammalian vertebrates have reported the presence of Ig-like immune receptor families in birds, amphibians and fish (30). Novel immune-type receptors (NITRs) are present in a large number of teleost fish species, are encoded by multigene families and share structural and signaling similarities with mammalian KIR receptors (31, 32). Teleost novel immunoglobulin-like transcripts (NILTs) share structural similarities with mammalian TREM and NKp44 receptors (33, 34). Modular

domain immune-type receptors (MDIRs) from cartilaginous fish (clearnose skate) and zebrafish share stuctural similarities with mammalian CD300, TREM/TLT, FCAR and pIgR receptors (18, 35). We report the structural and functional characterization of a Soluble Immune-Type Receptor (SITR) in a teleost fish. Carp SITR is a type I protein with two extracellular Ig domains in a rare organisation of a N-proximal V/C2 (or I-) type Ig domain and a C-proximal V-type, devoid of a transmembrane domain or any intracytoplasmic motif. The V-type Ig domain of SITR shows clear sequence homology to mammalian vertebrate CD300 molecules. Carp SITR is expressed abundantly in macrophages and is secreted upon stimulation with the protozoan parasite *Trypanoplasma borreli*. Carp SITR promotes PKC-dependent NO production in RAW macrophages and is involved in *T. borreli*-induced iNOS gene expression in carp macrophages.

MATERIALS AND METHODS

Ethic statement

All animals were handled in strict accordance with good animal practice as defined by the relevant national and/or local animal welfare bodies, and all animal work was approved by the animal experimental committee of Wageningen University, Wageningen, The Netherlands. (license numbers: 2004079/2004137/2008054)

Animals

European common carp (*Cyprinus carpio carpio* L.) were reared in the central fish facility of Wageningen University, The Netherlands at 23 °C in recirculating UV-treated tap water and fed pelleted dry food (Skretting, Nutreco) daily. R3xR8 carp are the hybrid offspring of a cross between fish of Hungarian origin (R8 strain) and of Polish origin (R3 strain)(36). Carp were between 9 and 11 months old at the start of the experiments.

Parasites

Trypanoplasma borreli was cloned and characterized by *Steinhagen et al.*(37). Parasites were maintained by syringe passage through carp. Parasitaemia was monitored in 10 x diluted blood in cRPMI [RPMI 1640 (Invitrogen, CA, USA) adjusted to carp osmolarity 280 $mOsmkg^{-1}$ containing 50 U/ml of heparin (Leo Pharma BV, Weesp, The Netherlands)] using a Bürker counting chamber. The minimum detection limit by this method was 10^5 parasites/ml of blood. For parasite isolation, blood was collected from 3-weeks-infected fish and purified on a 1x12cm ion-exchange chromatography using DEAE cellulose (DE-52; Whatman international) (38). After purification, parasites were resuspended in HML medium (39) supplemented with 5% pooled carp serum, L-glutamine (2 mM, Cambrex, Verviers, Belgium), penicillin G (100 U/ml, Sigma-Aldrich), and streptomycin sulfate (50 mg/l, Sigma-Aldrich).

Reagents

Inducer of retrograde protein transport Brefeldin A (BFA) from *Penicillium brefeldianum* and LPS from *Escherichia coli* were purchased from Sigma-Aldrich (St. Louis, MO). Inhibitor of endosomal acidification chloroquine and inhibitor of phosphatidylinositol 3-kinase (PI3K) LY294002 were purchased from InvivoGen (Cayla SAS, France). Syk tyrosine kinase inhibitor Piceatannol was purchased from Bio-connect (Tocris Biosciences, Missouri, USA) and Src tyrosine kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine (PP2) was purchased from Gibco (Invitrogen, CA, USA). Protein kinase C (PKC) inhibitor Staurosporine was purchased from Alexis Biochemicals (San Diego, CA, USA).

Generation of a subtracted cDNA library

A subtracted cDNA repertoire was generated by Suppression Subtraction Hybridization (SSH) using the PCR (polymerase chain reaction)-Select cDNA subtraction kit Catalog no. 637401 (Clontech, Palo Alto, CA). cDNA from macrophages stimulated with live *T. borreli* parasites for 6h was used as tester and cDNA from unstimulated macrophages was used as a driver. Tester and driver samples represent a pool of cDNA samples from n=4 fish. The subtracted cDNA repertoire was amplified by PCR according to the manufacturers protocol of the PCR-Select cDNA subtraction kit (Clontech). The resulting PCR products were ligated and cloned into JM-109 cells using pGEM-Teasy kit (Promega) according to the manufacturer´s protocol. Two hundred and eighty-eight clones were picked and sequenced. A nucleic-acid homology search revealed that out of 288 clones, 3 clones represented the SITR molecule described in this manuscript.

Amplification of carp SITR full-length cDNA

Oligonucleotide primers for carp Soluble Immune-Type Receptor (SITR) were designed based on the (partial) consensus sequence of 274bp obtained by SSH. cDNA from macrophages stimulated with *T. borreli* for 6h was used as template for PCR or nested PCR. The 5´and 3´ends of SITR were amplified using gene-specific primers (Forward SITR: ATTTCAGTCGGATTTTGGCTCAG and Reverse SITR: CGTAGCTTTCAACACCTAAACTGAGC) by 5´and 3´rapid amplification of cDNA ends (RACE) using the Gene Racer™ RACE Ready cDNA kit (Invitrogen, Breda, The Netherlands) according to the manufacturer´s protocol. PCR reactions were performed in *Taq* buffer, using 1U *Taq* polymerase (Promega, Leiden, The Netherlands) supplemented with $MgCl_2$ (1.5 mM), dNTPs (200 µM) and primers (400 nM each) in a total volume of 50 µl. PCR and nested PCR were carried out under the following conditions: one cycle 4 min at 96 °C; followed by 35 cycles of 30 sec at 96 °C, 30 sec at 55 °C and 2 min at 72 °C; and final extension for 7 min at 72 °C, using a GeneAmp PCR system 9700 (PE Applied Biosystems, Foster City, CA). Products amplified by PCR, nested PCR or RACE-PCR were ligated and cloned in JM-109 cells using the pGEM-Teasy kit (Promega) according to the manufacturer´s protocol. From each product both strands of eight clones were sequenced, using the ABI prismBigDye Terminator Cycle Sequencing Ready Reaction

kit and analysed using 3730 DNA analyser.

Bioinformatic analysis of carp SITR sequence

Nucleotide sequence was translated using the ExPASy translate tool (http://us.expasy.org/tools/dna.html) and aligned with Clustal W (http://www.ebi.ac.uk/clustalw). The signal peptide cleavage site and the transmembrane region was predicted by using the SignalP 3.0 (http://www.cbs.dtu.dk/services/SignalP/) and the TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/) servers, respectively. Subcellular location was predicted using the TargetP (http://www.cbs.dtu.dk/services/TargetP/) server. Post-translational modifications were predicted using the NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/), the NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/) and the NetPhospho K1.0 (http://www.cbs.dtu.dk/services/NetPhosK/) servers. Homology searches were performed using blastp http://www.expasy.org/tools/blast/ and the WU-Blast http://www.proweb.org/Tools/WU-blast.html servers. Identification of protein domains were predicted using PFAM (http://pfam.sanger.ac.uk/) and SMART (http://smart.embl-heidelberg.de/) servers. Prediction of β-strands for each Immunoglobulin domain was predicted using Swissmodel (http://swissmodel.expasy.org/) and PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/) servers.

Zebrafish SITR sequences retrieval and phylogenetic analysis

Chromosomes (Chr) within the zebrafish genome database were searched by basic local alignment search tool (BLAST) analysis (40) using the amino acid sequences for the carp SITR protein. Subsequently, the DNA surrounding homologues of this gene was retrieved (~400,000 bp) for further analysis with various sequence software programs. Using Genscan (41) possible coding regions within the genomic DNA were identified and the amino acid sequences analysed using BLAST (40) and FASTA (42). Phylogenetic relationships were constructed from ClustalX v1.81 (43) generated alignments of the full-length amino acid sequences of the known SITR-related molecules using the Neighbor-joining method (44). The tree was drawn using TreeView v1.6.1 (45)[Page] and confidence limits added (46).

Macrophage cell culture

Head kidney-derived macrophages, considered the fish equivalent of bone marrow-derived macrophages, were prepared as previously described (47, 48). Briefly, carp head-kidneys were gently passed through a 100 µm sterile nylon mesh (BD Biosciences, Breda, The Netherlands) and rinsed with homogenization buffer [incomplete-NMGFL15 medium containing 50 U/ml penicillin G, 50 µg/ml streptomycin sulphate, and 20 U/ml heparin (Leo Pharmaceutical, Weesp, The Netherlands)]. Cell suspensions were layered on 51% (1,07 $g.cm^{-3}$) Percoll (Amersham Biosciences, Uppsala, Sweden) and centrifuged at 450 g for 25 min at 4 °C with the brake disengaged. Cells at the interphase were removed and washed twice in incomplete NMGFL-15 medium. Cell cultures were initiated by seeding 1.75 x 10^7 head kidney leukocytes in a 75 cm^2 culture flask containing 20 ml of complete NMGFL-15

medium [incomplete-NMGFL-15 medium supplemented with 5% heat-inactivated pooled carp serum and 10% fetal bovine serum]. Head kidney-derived macrophages, named macrophages throughout the manuscript, were harvested after 6 days of incubation at 27 °C by placing the flasks on ice for 10 min prior to gentle scraping.

Gene expression analysis

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Leusden, The Netherlands) including the accompanying DNase I treatment on the columns, according to the manufacturers´ protocol and stored at -80°C until further use. Prior to cDNA synthesis, a second DN*ase* treatment was performed using DN*ase* I, Amplification Grade (Invitrogen). Synthesis of cDNA was performed with Invitrogen's SuperScript™ III First Strand Synthesis Systems for RT-PCRusing random primers according to the manufacturer's instructions. A non-reverse transcriptase control was included for each sample. cDNA samples were further diluted 50 times in nuclease-free water before use as template in real-time PCR experiments. Real time quantitative PCR (RT-qPCR) was performed in a 72-well Rotor-Gene™ 6000 (Corbett Research, Mortlake, Sydney, Australia) with the Brilliant® SYBR® Green QPCR (Stratagene, La Jolla, CA, USA) as detection chemistry as previously described (48). The primers used for RT-qPCR are listed in Table I. Fluorescence data from RT-qPCR experiments were analysed using Rotor-Gene version 6.0.21 software and exported to Microsoft Excel. The cycle threshold C_t for each sample and the reaction efficiencies (E) for each primer set were obtained upon Comparative Quantitation Analysis from the Rotor-Gene version 6.0.21 software. The relative expression ratio (R) of a target gene was calculated based on the E and the C_t deviation of sample versus control (49, 50), and expressed relative to the S11 protein of the 40S subunit as reference gene .

Table I. Primers used for real-time quantitative PCR analysis

Primer	Sequence (5'-3')	GenBank Accession No.
40S Fw	CCGTGGGTGACATCGTTACA	AB012087
40S Rv	TCAGGACATTGAACCTCACTGTCT	
SITR Fw	GCTCCTGATGTGT+CTGTGGTGA[a]	HM370297
SITR Rv	CTCC+CCACTGTG+TAACAGC[a]	
iNOS Fw	AACAGGTCTGAAAGGGAATCCA	AJ242906
iNOS Rv	CATTATCTCTCATGTCCAGAGTCTCTTCT	
IL-1β Fw	AAGGAGGCCAGTGGCTCTGT	AJ245635
IL-1β Rv	CCTGAAGAAGAGGAGGAGGCTGTCA	

[a] The "+" is before the nucleic acid in which the locked nucleic acid was placed

Morpholino delivery in carp macrophages

A morpholino knockdown approach was used to knock-down SITR protein expression in carp macrophages by inhibition of SITR mRNA translation according to the manufacturer's instructions (Gene Tools, LLC, Philomath, USA). Two antisense morpholino (Gene Tools) were designed: SITR_morpholino_A (mo_A) and SITR_morpholino_B (mo_B). mo_A (5'-

TCTTCGTGTAGGAGGCCATTTCTTT-3') targets the carp SITR mRNA at positions -6 to +19 with respect to the ATG. mo_B (5'-CTCTTTGCTGATGTTTCCTGTAAGA-3') targets the carp SITR mRNA at positions -32 to -7 with respect to the ATG. As a negative control, we used a standard control (mo_ctrl, 5'-CCTCTTACCTCAGTTACAATTTATA-3') which was expected to have no target and no biological activity in carp macrophages (Gene Tools). The standard control was fused to carboxyfluorescein to estimate the efficiency of morpholino delivery. Carp macrophages were resuspended in rich-NMGFL-15 medium [incomplete-NMGFL-15 medium supplemented with 2.5% heat-inactivated pooled carp serum and 5% fetal bovine serum] and mo_control (5μM), mo_A (5μM), mo_B (5μM) and Endo-Porter (6μM) were added. The mix was immediately swirled, and after 48h of incubation, the efficiency of morpholino activity was tested by means of intracellular staining and western blot analysis of protein expression and by gene expression analysis.

Primary Antibodies

Mouse monoclonal antibody WCL-15 strongly reacts with the cytoplasm of carp monocytes and macrophages in tissue sections (51). Mouse monoclonal antibody TCL-BE8 binds to carp neutrophilic granulocytes (strong affinity), monocytes (low affinity) (52). Mouse monoclonal antibody WCI-12 binds to the heavy chain of IgM in carp B cells (53, 54). Mouse monoclonal antibody WCL6 recognizes a 90KDa membrane molecule on carp thrombocytes (55). Mouse monoclonal anti-phosphotyrosine antibody and rabbit IgG anti-β-tubulin antibody were purchased from Abcam (Cambridge, UK).

Polyclonal rabbit antibodies anti-carp SITR were produced against each of two synthetic peptides coupled to keyhole limpet hemocyanin (KLH), according to a 3-months standard protocol (Eurogentec S.A., Seraing, Belgium). For peptide 1, amino acids 45-60 (CYYDKKYTQQKKYWYS) and for peptide 2, amino acids 169-185 (YTVGRTDTSQNSSVQIS) of the carp SITR protein were chosen for immunization. Each of the peptide is present on a different Ig domain of the SITR protein. Affinity purification of rabbit IgG was performed against purified peptides and specificity assessed by ELISA (Eurogentec). Anti-SITR antibody produced against peptide 1 binds to a 28KDa protein as assessed by western blot analysis but, in contrast to anti-SITR produced against peptide 2, does not lead to any positivity when assessed by flow cytometer or immunohistochemistry. For this reason we measured SITR protein expression using anti-SITR antibody produced against peptide 2 and used the remaining anti-SITR antibody as isotype control.

Magnetic activated cell sorting (MACS)

Macrophage-enriched fractions of head kidney leukocytes were obtained essentially as previously described (48). Cell suspensions were layered on a discontinous Percoll gradient (1.020, 1.060, 1.070 and 1.083 g cm^{-3}) and centrifuged 30 min at 800 *g* with the brake disengaged. Cells at 1.070 and 1.083 g cm^{-3} were collected, pooled and washed twice with cRPMI [RPMI 1640 adjusted to carp osmolarity 280 $mOsmkg^{-1}$]. The monoclonal antibody TCL-BE8 (1:50) was used to separate neutrophilic granulocytes from macrophages by

MACS as previously described. Briefly, after incubation for 30 min with TCL-BE8 on ice, the leukocyte suspension was washed twice with cRPMI and incubated with phycoerythrin (PE)-conjugated goat anti-mouse (1:50; DAKO, Glostrup, Denmark) for 30 min on ice. The magnetic separation was performed on LS-MidiMACS Columns (Mitenyi Biotec) according to the manufacturer's instructions. The purity of the TCL-BE8$^+$ (neutrophilic granulocyte-enriched fraction; > 90%) and TCL-BE8$^-$ (macrophage-enriched fraction; < 10%) fractions was confirmed by flow cytometric analysis using a FACScan® flow cytometer (Becton Dickinson, Mountain View, CA, USA).

Western blot analysis

Carp macrophages or mouse RAW macrophages were resuspended by pippeting and transferred to pre-cooled eppendorf tubes. Cells were washed twice in ice-cold PBS, lysed on ice with lysis solution [0.5% Triton X-100, 20 mM Tris, 100 mM NaCl, 1 mM EDTA, 50 mM NaF (Sigma), 5μM Na_3VO_4 (Sigma) 1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma)], homogenized with a syringe and incubated 10 min on ice. Cell lysates were centrifuged at 21000 *g* for 10 min at 4°C. Supernatant was collected and total protein content was determined by the Bradford method. Samples (20-25 μg) were boiled at 96 °C for 10 min with loading buffer containing β-mercaptoethanol and separated by 10% or 12.5% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes (Protrans, Schleicher & Schuell, Bioscience GmbH). Membranes were blocked in 5% w/v milk powder in TBS-T (10 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.5) for 1 h at room temperature and then incubated with primary antibody overnight at 4°C in 5% w/v BSA in TBS-T. Following antibodies were used: polyclonal rabbit anti-SITR antibody (1:100), mouse monoclonal anti-phosphotyrosine antibody (1:500) and rabbit IgG anti-β-tubulin (1:500). Membranes were then incubated with goat-anti-mouse HRP-conjugated (1:1000, Dako, Glostrup, Denmark) or goat-anti-rabbit HRP-conjugated (1:2000, Dako) in 5% w/v milk powder in TBS-T for 1 h at room temperature. Between each incubation step, membranes were washed three times in TBS-T for 10 min at RT. Signal was detected by development with a chemoluminescence kit (Amersham) according to the manufacturer's protocol and visualized by the use of Lumni-fil chemiluminescent Detection Film (Roche, Woerden, The Netherlands).

Intracellular SITR staining

For intracellular staining of SITR in carp macrophages, $1x10^6$ cells were resuspended in 50μL FACS buffer (0.5% BSA, 0.01% in PBS) and transferred to a 96-well U-bottom plate. For intracellular staining of SITR in mouse RAW macrophages, $0.25x10^6$ cells were resuspended in 50μL FACS buffer and transferred to a 96-well U-bottom plate. Following steps were perfomed on ice unless stated otherwise. Cells were first incubated 20min with blocking solution (10% foetal bovine serum in PBS) to reduce non-specific immunofluorescent staining. After washing with FACS buffer, cells were permeabilized by incubation for 15min with 100μL Cytofix/Cytoperm (BD Bioscience, California, USA). After a washing step

with 1x Perm/Wash buffer (BD Bioscience), cells were incubated for 30min with affinity-purified polyclonal rabbit anti-SITR antibody or with the isotype control (both at 1:10 in Perm/Wash buffer). After washing 1x Perm/Wash buffer, cells were incubated for 30min in the dark with the swine-anti-rabbit antibody conjugated with fluorescein isothiocyanate (SWAR-FITC, 1:50, Dako) as secondary antibody. After extensive washing with 1x Perm/ Wash buffer, cells were transferred to flow cytometer tubes. Fluorescent intensities of 10^4 events were acquired in log scale using a Beckman Coulter Epics XL-MCL flow cytometer. Incubation with the isotype control (anti-SITR antibody produced against peptide 1) lead to no positive reaction.

Immunohistochemistry

Cryosections (7 µM) of spleen tissue were mounted on poly-*L*-lysine-coated glass slides (BDH Laboratory Supplies, Poole, UK), air-dried for 60 min and incubated in a 0.3 % H_2O_2 solution in methanol for 20 min to inactivate endogenous peroxidase. Following steps were performed at RT unless stated otherwise. Cryosections were washed for 5 min with PBS, then short with distilled water and incubated in proteinase-K solution (50 µg/ml in distilled water) for 10 min at 37 °C. Samples were fixed in 4 % paraformaldehyde in PBS for 10 min at 4 °C followed by washing in 0.1 % Triton PBS (PBS-T) for 10 min at 4 °C and subsequently in PBS-T for 7 min at RT. A blocking solution of 5% normal goat serum was then added onto the slides and incubated for 30min. Affinity-purified polyclonal rabbit anti-SITR antibody (1:10) was then added alone or in combination with the mouse monoclonal antibodies WCL-15 (1:50), TCL-BE8 (1:50), WCI-12 (1:50) or WCL-6 (1:50) in PBS for 1h. After washing twice for 10min in PBS-T, sections were incubated with the secondary antibody for 1h with goat anti-rabbit antibody conjugated to alkaline-phosphatase (GAR-AP, Dako, 1:150 in PBS) alone or in combination with goat anti-mouse antibody conjugated to horseradish peroxidase (GAR-HRP, Dako, 1:150 in PBS). When only GAR-AP antibody was used, sections were first incubated in AP-buffer (0.1M Tris-Cl, 0.1M NaCl, 0.05M $MgCl_2$, pH 9.5) for 10min and then stained using AP substrate [4.5 µl/ml nitro-blue-tetrazoleum (Roche Applied Science) and 3.5 µl/ml 5'-bromo-4'-chloro-3'-indolyl phosphatase (BCIP; Roche Apllied Science) in AP buffer] for 2-5 min followed by four washes in distilled water. Alternatively, when both secondary antibodies were used (double-staining), sections were first AP stained as described above. After rinsing four times with distilled water, sections were incubated for 10 min in 0.05 M sodium acetate buffer, pH 5 and following addition of 0.4 mg/ml 3-amino-9-ethyl-carbazole (AEC; Sigma-Aldrich) in sodium acetate buffer containing 0.03 % H_2O_2 and incubated for 25 min. Finally, cryosections were rinsed four times in distilled water and embedded in Kaiser's glycerine gelatin (Merck, Darmstadt, Germany).

Confocal laser scanning microscopy

Cytospins on poly-*L*-lysine coated glass slides (BDH Laboratory supplies) of TCL-BE8 negative fraction (MACS sorted) or carp macrophages were made by fixing in 100%

alcohol and 99% acetic acid (10:1). Mouse monoclonal WCL-15 (1:50) antibody and /or polyclonal rabbit anti-SITR (1:10) antibody were used as primary antibodies. Goat anti-mouse antibody conjugated to fluorescein isothiocyanate (GAM-FITC, Dako, 1:50 in PBS) and goat anti-rabbit conjugated to tetramethylrhodamine-5-(and 6)-isothiocyanate (GAR-TRITC, Dako, 1:50 in PBS) were used as secondary antibodies. Sections were embedded in Vectashield Mounting medium (Vector Laboratories) and examined with a Zeiss LSM-510 laser scanning microscope. FITC (green) signal was excited with a 488 nm argon laser and detected using a band-pass filter (505-530 nm) and TRITC (red) signal was excited with a 543 nm helium-neon laser and detected using a long-pass filter (560 nm).

SITR-GFP and TLR2ΔTIR-GFP expression plasmids

The vivid color™pcDNA™6.2/C-EmGFP-GW/TOPO® (Invitrogen, catalog no. K359-20) expression vector combined with TOPO®cloning was used to fuse full-length SITR or TLR2ΔTIR (TLR2 truncated at TIR domain, (48) to EmGFP at the C-terminal end. Isolation of highly pure plasmid DNA suitable for transfection was performed using S.N.A.P.™ Midi Prep Kit (Invitrogen, catalog no. K1910-01) according to the manufacturer´s protocol. C-terminal fluorescent-tagged protein could be visualized using confocal microscopy.

Transient transfection of murine RAW 264.7 macrophages

RAW 264.7 cells were cultured in RPMI (Invitrogen, CA, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 50 U/ml penicillin G (Sigma-Aldrich) and 50 µg/ml streptomycin sulphate (Sigma-Aldrich). One day before transfection, RAW cells were seeded into tissue culture flasks to reach 60-70% confluence at the day of transfection. For transient transfection, 2.5 µg of the carp SITR-GFP or TLR2ΔTIR-GFP constructs was transfected into RAW by nucleoporation using nucleofactor™ solution V and program D-32 (Lonza Cologne AG, Germany) according to the manufacturer´s instructions. For western blot analysis, 24hours after transfection, cells were scraped, counted using Trypan blue exclusion and plated overnight at a concentration 5 x 10^5 cells/ well in a 24-well tissue culture plate. The next day, cells were stimulated with 5 x 10^5 live *Trypanoplasma borreli* parasites for 15 min or left untreated as control and lysed for further analysis. For measurement of NO production, 24hours after transfection, cells were scraped, counted using Trypan blue exclusion and plated overnight at a concentration 2 x 10^5 cells/ well in a 96-well tissue culture plate. The next day, 75 µL supernatant was collected and nitrite production was measured.

Nitrite production

Nitrite production was measured essentially as described before (56): to 75 µl of cell culture supernatant, 100 µl of 1% sulfanilamide in 2.5% (v/v) phosphoric acid and 100 µl of 0.1% (w/v) *N*-naphthyl-ethylenediamine in 2.5% (v/v) phosphoric acid were added in a 96-well flat-bottom plate. The absorbance was read at 540 nm (with 690 nm as a reference) and nitrite concentration (µM) was calculated by comparison with a sodium

nitrite standard curve.

Statistical Analysis

Transformed values (ln) were used for statistical analysis in SPSS software (version 17.0). Homogeneity of variance was analyzed using the Levene's test. Significant differences between treatments ($P \leq 0.05$) for the *in vitro* studies were determined by one-way ANOVA followed by Sidak's test. In case of unequal variances between treatments, the one-way ANOVA was followed by a Games–Howell test.

RESULTS

Cloning and sequence analysis of carp Soluble Immune-Type Receptor SITR

A subtracted cDNA repertoire from common carp macrophages, enriched for genes up-regulated in response to the protozoan parasite *T. borreli*, was generated by SSH. Included in the repertoire (3/300 positive clones) was a partial consensus sequence for a novel immune-type receptor. Specific primers based on the initial sequence amplified a full-length cDNA sequence of 1114 bp with an open reading frame of 723 bp, encoding for a protein of 241 aa with a predicted molecular weight of 27.4 KDa.

The novel cDNA has two predicted Ig-like domains and a 15 aa short proximal C-terminal region. Although the 15 aa proximal C-terminal region appears rich in positively charged amino acids (K232, K234, R236), the protein has no clear transmembrane region nor structural hallmarks suggestive of stimulatory or inhibitory (e.g.: ITAM, ITIM) signalling potential (Fig. 1A). The novel protein has a putative *N*-glycosylation site at position 73 and several Serine (13 sites), Threonine (4 sites) and Tyrosine (6 sites) phosphorylation sites as well as a protein kinase C (PKC) phosphorylation site at position T65 (Fig. 1A). Sequence analysis classifies it as a type I soluble protein with a putative hydrophobic 24-aa signal peptide expected to induce secretion. No vacuolar targeting signal was predicted. Thus, structural analysis of the novel protein sequence suggests SITR to be a secreted protein.

Amino acid sequence alignment of the two Ig-like domains (Fig. 1B-C) showed a high degree of similarity (BLAST *E* value $\leq 10^{-9}$) of the C-proximal Ig-like domain (Fig. 1C) with Ig domains from mouse CMRF-35-like molecules (CLM) and human CD300 orthologues as well as with mammalian polymeric immunoglobulin receptor (pIgR) molecules. In contrast, the degree of similarity of the N-proximal Ig-like domain (Fig. 1B) with these mammalian Ig domains was low (BLAST *E* value $\leq 10^{-4}$). Most CD300 Ig domains are characterized by the presence of two pairs of cysteine residues and typical aa motifs contained in strands forming the two β-sheets of the Ig domain. The novel protein has a C-proximal Ig-like domain with two pairs of cysteine residues and a conserved WCR motif in strand C and conserved FTV motif in strand E (Fig. 1C). In contrast, the N-proximal Ig-like domain has a single pair of cysteine residues only,

with a conserved tryptophan (W) motif in strand C and a conserved FTVT motif in strand E (Fig. 1B).

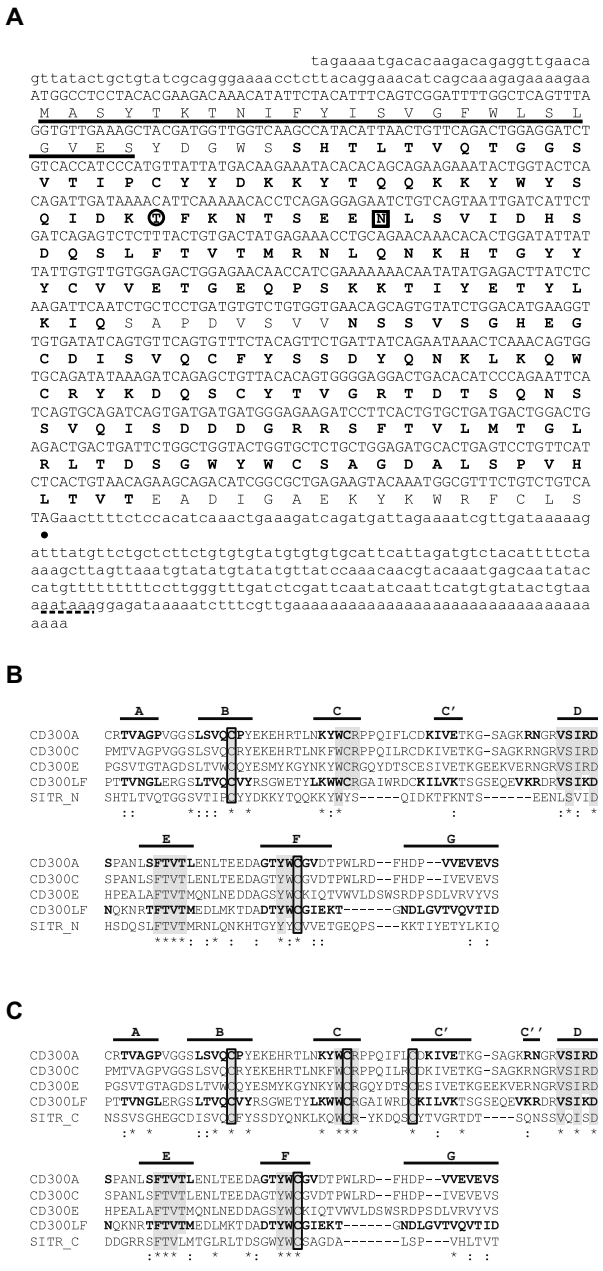

Figure 1. Carp Soluble Immune-Type Receptor (SITR) is a member of the Ig superfamily. A. Nucleotide sequence of common carp SITR with open reading frame (upper case) and untranslated 5' and 3' regions (lower case). The predicted amino acid sequence is shown below the nucleotide sequence. The predicted signal peptide is underlined and the two Ig-like domains are marked in bold. The potential *N*-glycosylation site is boxed and the potential PKC interaction site is circled. Dot indicates the stop codon. A consensus polyadenylation signal (AATAAA) in the 3'-UTR is dotted. B. Alignment of the putative carp SITR N-proximal Ig-like domain (SITR_N, residues 30-123) with V-type Ig domains from human CD300 molecules. C. Alignment of the putative carp SITR C-proximal Ig-like domain (SITR_C, residues 132-224) with V-type Ig domains from human CD300 molecules. Asterisks indicate identity and colons denote similarity. Dashes indicate the introduced gaps to maximize the alignment. Residues characteristic of the V-type CD300 Ig-like fold and conserved between carp SITR (GenBank Accession Number: HM370297, http://www.ncbi.nlm.nih.gov/genbank/) and human CD300A (GenBank acc no: NP_009192.2), CD300C (GenBank acc no: NP_006669.1), CD300E (GenBank acc no: NP_852114.1), CD300F (GenBank acc no: NP_620587.2) are grey shaded. Conserved cysteines are boxed. Regions of β-strands, as defined by X-ray crystallography for CD300A (PDB acc no: 2Q87, http://www.rcsb.org/pdb/home/home.do) and CD300LF (PDB acc no: 2NMS) are marked in bold. The positions of the predicted β-strands for carp SITR are indicated above the sequence.

Prediction of the β-strands using Swiss Model and PSIPRED databases and sequence alignment with β-strand regions defined by X-ray crystallography of CD300A and CD300 LF molecules, defined a long spacing between the putative β-strands C and D, suggesting the presence of additional β-strands (e.g.: C' and C''). The prediction servers confirmed the presence of a C' strand for both Ig-like domains and possibly a C'' strand for the C-proximal Ig-like domain. Thus, we predict the N-proximal Ig-like domain to be of the V/C2- (or I-) type and predict the C-proximal Ig-like domain to be of the V-type. (GenBank Accession Number: HM370297, http://www.ncbi.nlm.nih.gov/genbank/).

In conclusion, sequence analysis suggests that the novel cDNA is a new member of the Ig-SF with a V/C2 type N-proximal Ig domain and a V-type C-proximal Ig domain. The V-type Ig domain displays homology with mammalian CD300 molecules and with modular domain immune type receptors (MDIRs) of the cartilaginous skate and zebrafish which are multigenic families of activating/inhibitory receptors. We conclude that we have identified, in common carp, a novel soluble immune-type receptor without typical activating/inhibiting characteristics named Soluble Immune-Type Receptor SITR.

Carp SITR displays a high basal expression

The SITR cDNA was initially identified in an SSH repertoire from carp macrophages stimulated with the protozoan parasite *T. borreli*. Studies of basal SITR gene expression in carp macrophages revealed a highly abundant expression in naïve macrophages similar to the level of the house keeping 40S ribosomal S11 protein gene (Fig. 2A). Gene expression in carp macrophages could be 2-fold upregulated, approximately, by the protozoan parasite *T. borreli* (Fig. 2B).

To study protein expression, we affinity-purified an antibody raised in rabbit against a peptide in the C-proximal Ig-domain of SITR (YTVGRTDTSQNSSVQIS). Two discrete bands could be shown in western blot, of which one with predicted 28 KDa MW (Fig. 2C). Pre-incubation of the anti-SITR antibody with the immunizing peptide resulted in a partial disappearance of the 45KDa but complete disappearance of the band with predicted molecular weight of 28 KDa of SITR, confirming specificity of the antibody.

Sequence analysis showed that, although the 15 aa proximal C-terminal region of SITR appears rich in positively charged amino acids, the protein has no clear transmembrane region and has a secreted function. To study the sublocalization of the protein in carp macrophages, we performed both surface and intracellular staining using the anti-SITR antibody. Lack of surface staining (Fig. 2D) supports the sequence analysis that carp SITR has no transmembrane region nor plasma membrane anchoring by, for example, GPI modifications. Clear intracellular staining (Fig. 2D) of 50-70 % of cells indicated that the majority of naïve carp macrophages express the SITR protein. As

a negative control anti-SITR antibody was pre-incubated with the immunizing peptide, which reduced intracellular staining to 20% only. Our results indicate that carp SITR is a soluble immunoglobulin-type receptor abundantly expressed intracellularly in naïve carp leukocytes.

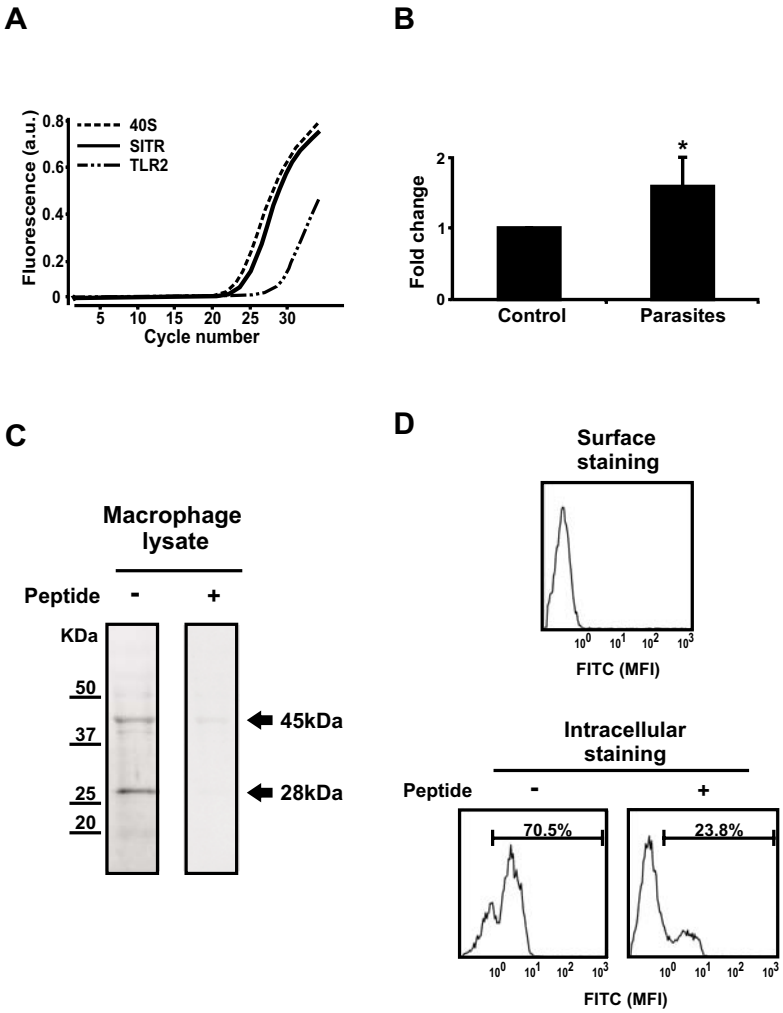

Figure 2. SITR gene and protein expression in carp macrophages.
A. Real-time qPCR cycle profile for SITR in naïve carp macrophages in comparison with the house keeping gene 40S ribosomal protein S11 and Toll-Like Receptor (TLR)2 as reference genes. B. SITR gene expression in carp macrophages after stimulation for 6 h with live *T. borreli* protozoan parasites (0.5 x 10^6 per well). mRNA levels of SITR relative to the house keeping gene 40S ribosomal protein S11 are expressed as fold change relative to unstimulated cells (control). Bars show averages ± SD of n=4 fish. Symbol (*) shows a significant ($P \leq 0.05$) difference compared with unstimulated cells. C. Western blot analysis of macrophage lysates using as primary antibody the anti-SITR antibody or the anti-SITR antibody pre-incubated with the immunizing peptide (20μg/ml). D. Surface and intracellular SITR protein staining detected by flow cytometry using anti-SITR antibody or anti-SITR antibody pre-incubated with the immunizing peptide (20μg/ml).

Carp SITR is expressed mainly in myeloid cells

The SITR gene was detected in protozoan parasite-stimulated carp macrophages, whereas the presence of SITR protein in carp macrophages was confirmed by western blot and flow cytometry. To examine the putative presence of SITR protein in leukocyte cell types other than macrophages we performed double-staining using anti-SITR antibody (blue) in combination with monoclonal antibodies (red) recognizing (WCL-15^+) monocytes/macrophages, (TCL-$BE8^+$) neutrophilic granulocytes, (WCI-12^+) B-cells or (WCL-6^+) thrombocytes in spleen from naïve carp. Immunohistochemical analysis confirmed the abundant SITR protein expression particularly in splenic macrophages (WCL-15^+ $SITR^+$ double-positive cells display a dark-purple colour) but not in neutrophilic granulocytes, B cells and thrombocytes (Fig. 3A-F). To examine the putative presence of the SITR gene in leukocyte cell types other than macrophages we also performed real-time qPCR on cDNA from purified leukocyte cell populations. Gene expression analysis confirmed the abundant expression of SITR transcript in monocyte/macrophages, showed moderate SITR gene expression in neutrophilic granulocyte-enriched fractions and weak SITR gene expression in B cell-, T cell- and thrombocyte-enriched fractions (data not shown). This suggests that SITR is preferentially expressed in myeloid cell types. To verify the high SITR prevalence in macrophages, two further sources of carp macrophages were examined for SITR protein expression; macrophage-enriched MACS-sorted leukocytes from head kidney and head kidney-derived cultured macrophages. Head kidney is the hematopoietic organ equivalent to the mammalian bone marrow. WCL-15^+ macrophages displayed SITR+ positivity (Fig. 3G-H). The SITR protein was never detected on the cell surface membrane but localized intracellularly within macrophages in vesicle-like structures.

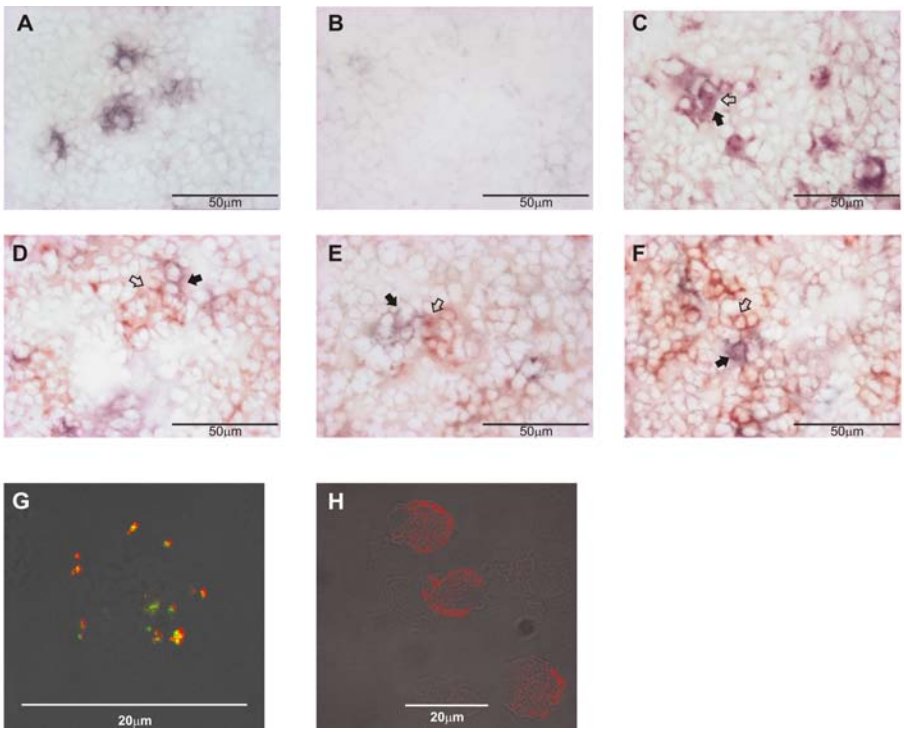

Figure 3. SITR protein is mainly expressed in myeloid cells.
A. Anti-SITR immunoreactivity (blue) in spleen of naïve carp. B. Anti-SITR immunoreactivity (blue) after pre-incubation of the anti-SITR antibody with the immunizing peptide (20μg/ml) in spleen of naïve carp. C. Double-staining for monocytes/macrophages (WCL-15; red) and SITR (blue) in spleen of naïve carp. D. Double-staining for neutrophilic granulocytes (TCL-BE8; red) and SITR (blue) in spleen of naïve carp. E. Double-staining for B cells (WCI-12; red) and SITR (blue) in spleen of naïve carp. F. Double-staining for thrombocytes (WCL-6; red) and SITR (blue) in spleen of naïve carp. G. Double-staining for monocytes/macrophages (WCL-15; green) and SITR (red) in macrophage-enriched cell fractions from head-kidney of naïve carp. H. Staining for SITR (red) in macrophage-enriched cell fractions from head-kidney of naïve carp. Typical red-stained cells are indicated with open arrows and typical blue-stained cells with closed arrows. Note that in panel C, it is difficult to distinguish between red- and blue-stained cells. Co-localization of both signals results in dark purple-stained cells.

Carp SITR is secreted upon stimulation with protozoan parasites

The SITR cDNA was initially identified to be differentially expressed in carp macrophages stimulated with *T. borreli*. To confirm SITR protein regulation by this protozoan parasite, macrophages were incubated with live *T. borreli* parasites. The presence of two distinct populations ($SITR^{dull}$ and $SITR^{high}$), corresponding to differences in SITR protein expression, was evident (Fig. 4A). Stimulation with live parasites did not have an effect on mean fluorescence intensity (MFI) but did result in a decrease in cell density of the $SITR^{high}$ population already after 15 min (0.25 hour; Fig. 4A). After 3 h stimulation with live parasites, the percentage of $SITR^{high}$ macrophages reduced

from 50% to 35%, approximately (Fig. 4B). Decrease of SITR protein expression increased in time after stimulation with protozoan parasites, but not after stimulation with unrelated ligands such as peptidoglycan or lipopolysaccharide (data not shown). Our results therefore suggest a ligand-specific secreted function for SITR in carp macrophages.

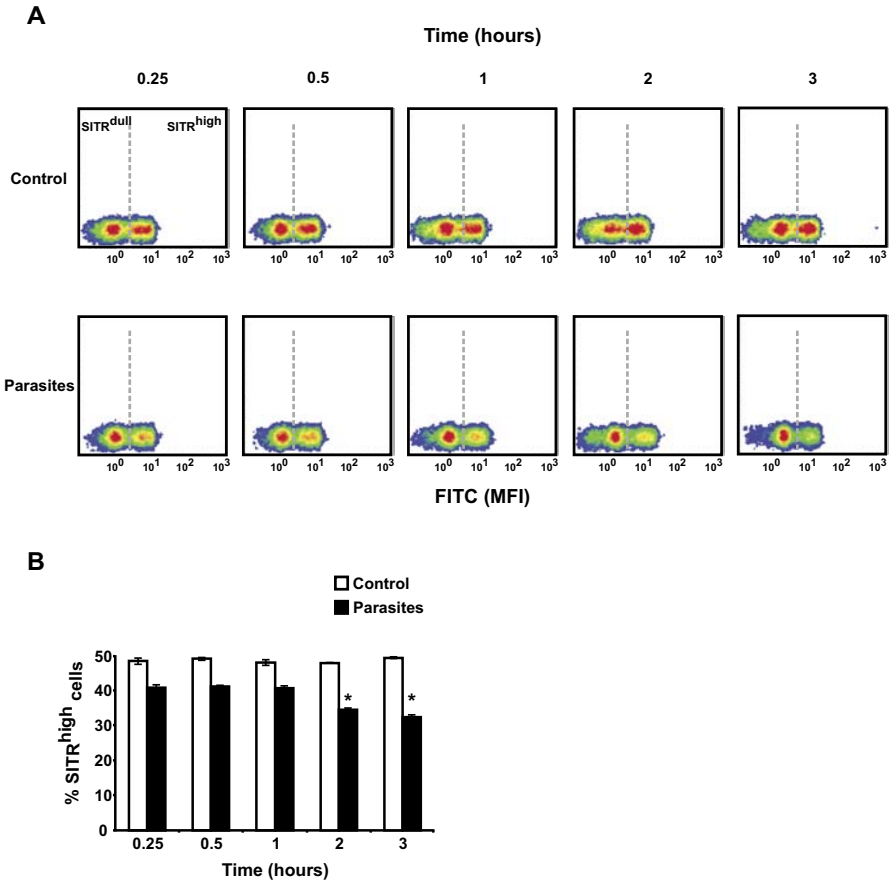

Figure 4. Effect of parasite stimulation on intracellular SITR protein expression.
A. Density plots of intracellular SITR protein expression analysed by flow cytometry using anti-SITR antibody. Macrophages were stimulated with live *T. borreli* parasites (0.5 x 10^6 per well) for different time periods or left untreated as negative control. Two populations of cells could be defined on the basis of SITR protein expression: $SITR^{dull}$ and $SITR^{high}$. Density plots shown are representative of one out of three experiments. B. Percentage $SITR^{high}$ cell populations (averages ± SD of n=3 fish) after stimulation of macrophages with live T. borreli parasites for different time periods, or left untreated as negative control. Symbol (*) indicates a significant (P≤ 0.05) difference in parasite-stimulated cells compared with unstimulated cells at the same time point.

Sequence analysis classified carp SITR as a type I soluble protein expected to be secreted extracellularly. To assess whether the observed decrease of intracellular

staining for SITR upon stimulation with live *T. borreli* should be ascribed to secretion of SITR protein, an optimized concentration of brefeldin A (BFA) was used. BFA is an inducer of retrograde protein transport from the Golgi to the ER, leading to protein accumulation in the ER and therefore impairs protein secretion. The percentage of SITR+ macrophages reduced from 65% to 45%, approximately, after incubation with only BFA (Fig. 5A, C). Stimulation of macrophages with both BFA and live *T. borreli* parasites increased intracellular SITR protein expression from 55% to 70%, approximately (Fig. 5B, C). The use of an inhibitor of endosomal acidification (chloroquine) did not impair the secretion of SITR protein upon parasite stimulation, confirming the targeting of the protein to the extracellular space and not to the lysosomal compartment (data not shown). Hence, our results strongly suggest that stimulation of macrophages with live protozoan parasites promotes the secretion of SITR protein *in vitro*.

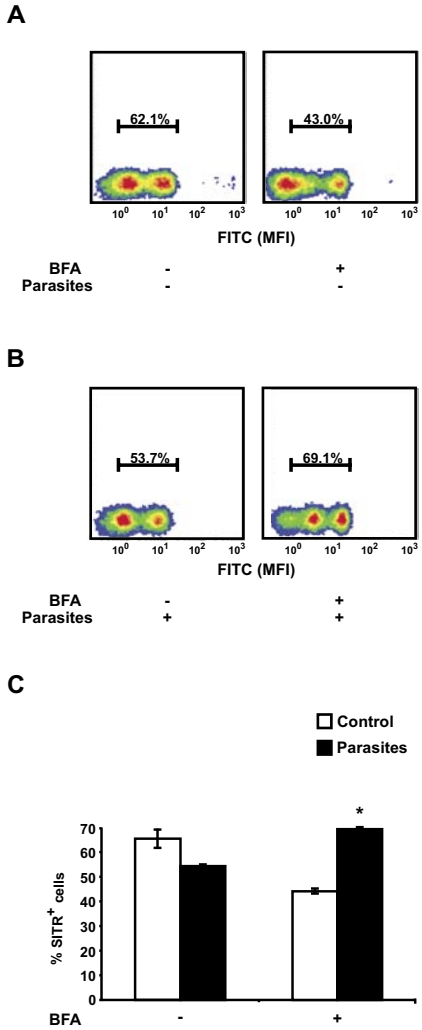

Figure 5. Intracellular SITR protein sorting.
A. Carp macrophages were incubated for 16.5 h with brefeldin A (BFA, 2 μg/ml) or left untreated as negative control. B. Macrophages were pre-incubated for 30 min with BFA (2 μg/ml) or left untreated as negative control and further stimulated for 16 h with live *T. borreli* parasites (0.5 x 10^6 per well). The density plots of intracellular SITR protein expression analysed by flow cytometry using anti-SITR antibody are representative for four experiments. C. Percentage $SITR^{high}$ cell populations (averages ± SD of n=4 fish) after pre-incubation for 30 min with BFA, or left untreated as negative control, followed by stimulation with live *T. borreli* parasites for 16 h. Symbol (*) indicates a significant ($P \leq 0.05$) difference in parasite stimulated cells compared to unstimulated cells.

Carp SITR is secreted during in vivo infection with the protozoan parasite T. borreli

In vitro stimulation of carp macrophages with *T. borreli* resulted in a decrease of intracellular staining suggestive of SITR secretion. Immunohistochemical analysis of spleen of *T. borreli*-infected carp showed a similar decrease of SITR protein expression at week 1-3 post-infection, increasing to basal levels at 5 weeks post-infection (Fig. 6A). The simultaneous (moderate) increase rather than decrease in number of (WCL-15+) monocytes/macrophages (Fig. 6B), proved that the decrease in SITR protein expression during infection was independent of the absolute number of macrophages present. These results show a *T. borreli*-induced secretion of SITR protein *in vivo*.

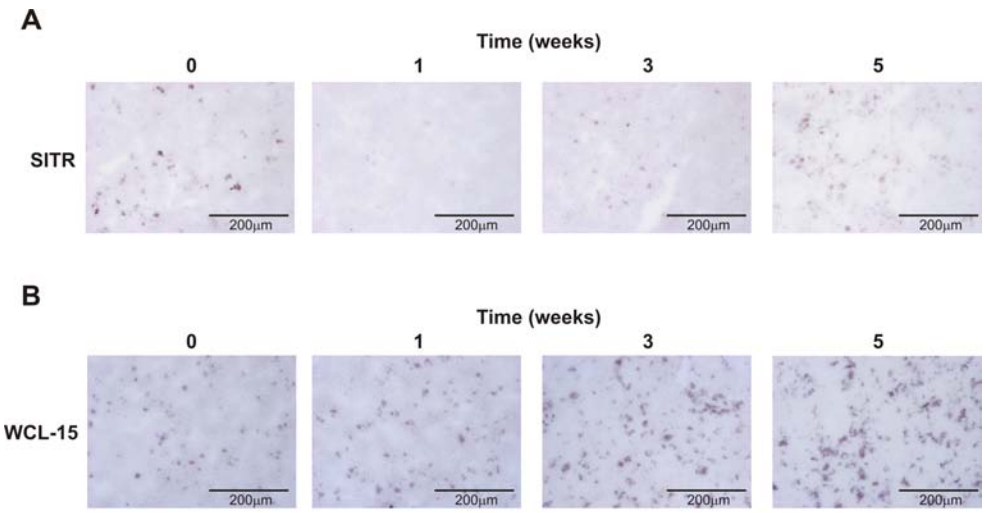

Figure 6. SITR protein expression during *in vivo T. borreli* infection.
A. Anti-SITR immunoreactivity (blue) in spleen tissue from non-infected fish (control) and at 1, 3 and 5 weeks post-infection with *T. borreli* parasites. B. Staining for onocytes/macrophages (WCL-15; red) in spleen tissue from non-infected fish (control) and at 1, 3 and 5 weeks post-infection with *T. borreli* parasites.

Overexpression of carp SITR in RAW cells increases NO production

To investigate macrophage functions associated with SITR activation, we transfected mouse (RAW) macrophages with a construct expressing the carp SITR gene. Transfection efficiency ranged between 25% and 35% as measured by intracellular staining using anti-SITR antibody (Fig. 7A). RAW cells transfected with carp TLR2 truncated at the TIR domain (TLR2ΔTIR), or non-transfected RAW cells, were used as negative controls. The SITR protein has several predicted tyrosine phosphorylation sites as well as a protein kinase C (PKC) phosphorylation site (see Fig. 1A), suggesting that it could be involved in tyrosine phosphorylation- and PKC-dependent mechanisms.

Total tyrosine phosphorylation was analysed by western blot using an anti-phospho tyrosine antibody. Stimulation of SITR-transfected RAW cells with *T. borreli* parasites resulted in an induction of tyrosine phosphorylation stronger than in negative controls (Fig. 7B). Nitrite production is one of the signature features of *T. borreli* infections in carp and is kinase dependent (57, 58) We observed an increase of basal NO levels in SITR-transfected RAW cells, but not in negative controls (Fig. 7C). Inhibition of Src and Syk kinases did not inhibit SITR-induced NO production in transfected RAW cells. In contrast, inhibition of PKC kinase, and to some extent inhibition of PI3K kinase, resulted in an abrogation of SITR-induced NO production (Fig. 7D). These results suggest that *T. borreli*-induced stimulation of SITR results in the activation of tyrosine phosphorylation dependent cascades, including a PKC-dependent route that leads to NO production.

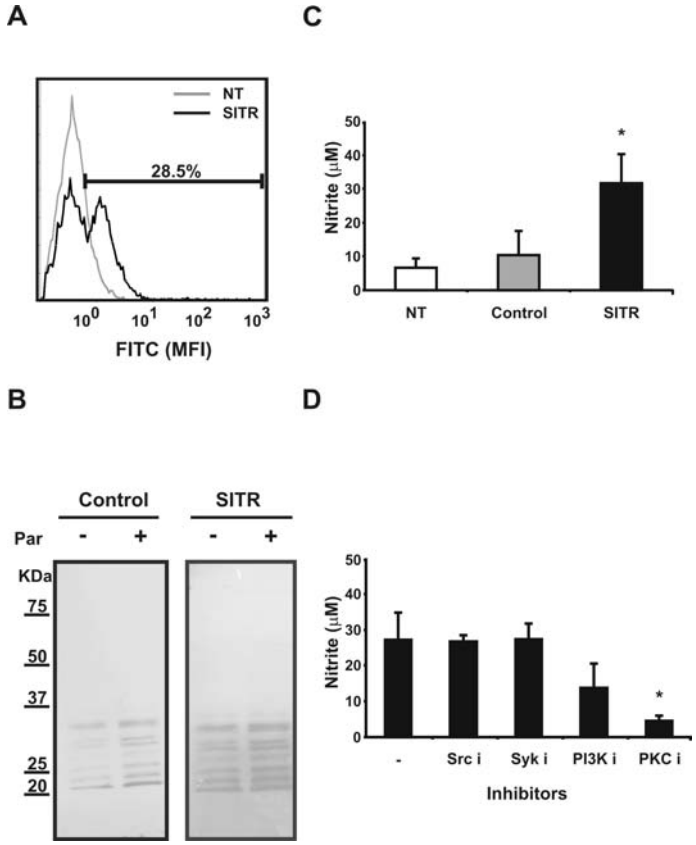

Figure 7. Overexpression of SITR in mouse RAW macrophages.
A. Intracellular SITR protein expression in RAW cells analysed by flow cytometry using anti-SITR antibody (1:50). RAW cells were non-transfected (NT) or transfected with carp SITR. B. Western blot of cell lysates of TLR2ΔTIR- (control) and SITR-transfected RAW cells stimulated with live *T. borreli* parasites (Par, 0.5 x 10^6 per well) for 15 min or left untreated as control. Tyrosine phosphorylation was evaluated using an anti-phospho tyrosine antibody. C. Nitrite concentration (averages ± SD of n=5) in supernatants of non-transfected (NT), TLR2ΔTIR-(control) and SITR- transfected RAW cells determined by Griess reaction at 24 h. Symbol (*) indicates a significant ($P \leq 0.05$) difference compared with TLR2ΔTIR- transfected RAW cells. D. Nitrite concentration (averages ± SD of n=3) in supernatants of SITR- transfected RAW cells pre-incubated for 30 min with inhibitors of Src kinase (Src i, PP2, 20μM), Syk kinase (Syk i, Piceatannol, 50μM), and PI3K kinase (PI3K i, LY294002, 50μM), PKC kinase (PKC i, Staurosporine, 1μM) or left untreated as control. TLR2ΔTIR-(control)- transfected RAW cells were used as negative controls (data not shown). Nitrite levels were determined by Griess reaction at 24h. Symbol (*) indicates a significant ($P \leq 0.05$) difference compared with unstimulated cells.

Knock-down of SITR in carp macrophages down-regulates gene expression of iNOS

Overexpression of carp SITR in RAW cells resulted in increased NO levels, suggesting a role for the SITR molecule in NO production. To verify SITR involvement in NO induction, antisense morpholinos were designed to knock-down SITR gene translation in carp macrophages. Use of a non-specific morpholino fused to 3'-carboxyfluorescein showed morpholino delivery was successful after 24 h, but maximal after 48 h. Successful inhibition of SITR translation was evaluated by intracellular staining and western blot with SITR antibody. Morpholino B, but not morpholino A nor the non-specific morpholino reduced the Mean Fluorescence Intensity (MFI) of SITR-positive macrophages (data not shown). Similarly, only use of morpholino B reduced the intensity of the 28 KDa SITR-specific band in western blot (Fig. 8A). Therefore, morpholino B was used to verify SITR involvement in NO induction by carp macrophages.

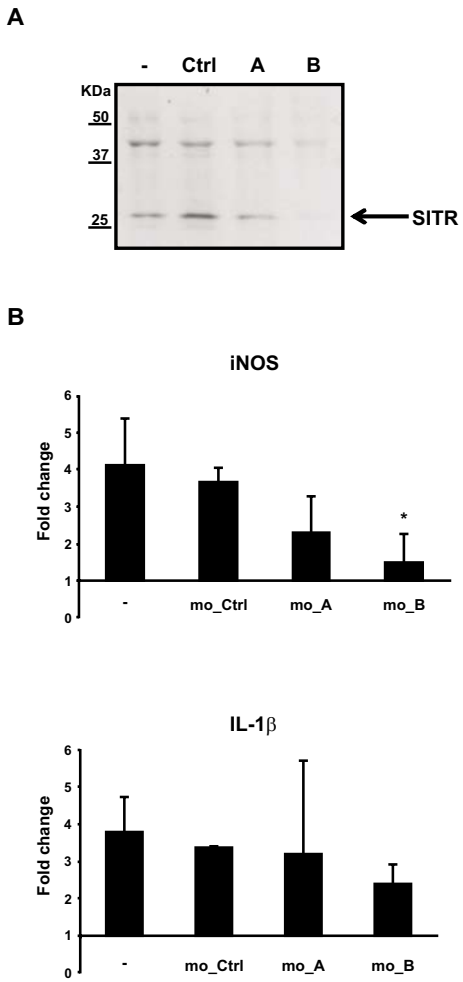

Figure 8. Knock-down of SITR protein in carp macrophages. A. Western blot of cell lysates from carp macrophages incubated for 48 h with control morpholino (control, 5μM), SITR morpholino A (SITR_A, 5 μM) or SITR morpholino B (SITR B, 5 μM) or left untreated as control. SITR protein expression was analysed using anti-SITR antibody. B. Real-time gene expression in carp macrophages pre-incubated for 48 h with control morpholino (mo_ctrl, 5 μM), SITR morpholino A (mo_A, 5 μM) or SITR morpholino B (mo_B, 5 μM) or left untreated. Macrophages were further stimulated for 6 h with live *T. borreli* parasites (0.5×10^6) or left unstimulated. mRNA levels of inducible nitric oxide synthase (iNOS) and Interleukin-1β (IL-1β) are shown relative to the house keeping gene 40S ribosomal protein S11 and are expressed as fold change relative to unstimulated cells (fold change=1). Bars show averages ± SD of n=4 fish. Symbol (*) shows a significant ($P \leq 0.05$) difference compared to macrophages incubated with control morpholino.

Stimulation of carp macrophages with live *T. borreli* parasites up-regulated gene expression of IL-1β and iNOS 5-fold approximately. Pre-incubation of carp macrophages with morpholino B, but not with non-specific morpholino, specifically reduced iNOS gene expression (Fig. 8B). These data show the involvement of SITR in protozoan parasite *T. borreli*-induced iNOS gene expression.

DISCUSSION

In this study, we describe the molecular cloning and functional characterization of a soluble immunoglobulin-like receptor SITR in teleost fish. Carp SITR has two extracellular Ig-domains with an unique organization: a V/C2 (or I-) type N-proximal Ig domain and a V-type C-proximal Ig domain. The carp SITR V-type Ig domain, in particular, has a close sequence similarity and conserved structural characteristics to mammalian CD300-like molecules. In contrast to the majority of IgSF receptors, SITR has no transmembrane domain. In carp, SITR is secreted by macrophages upon stimulation with protozoan parasites. Overexpression of SITR in mouse macrophages and knock-down of SITR in carp macrophages provides evidence for involvement of SITR in parasite-induced NO production.

Sequence analysis predicted carp SITR to have a N-proximal Ig-like domain of the V/C2- (or I-) type and a C-proximal Ig-like domain of the V-type. In general, Ig folds are formed by antiparallel β-strands arranged into two β−sheets linked by disulphide bonds and IgSF domains can be classified in V, C1, C2 and I types according to sequence patterns and length (3, 4). By convention, the β-strands have been labeled *A* to *G* (based on the C1 domain) with the two additional strands in V–type Ig domains, present between *C* and *D*, labeled *C*'and *C*''. One β-sheet consists of β-strands *A*, *B*, *E* and possibly C' and *C*'' while the other β-sheet contains strands *C*, *F*, *G*. Bork *et al.* (3) classified Ig-domains as belonging to V-types having all 9 β-strands, C1 types lacking the *C*' and *C*'', C2 types having the *C*' strand but not the *C*'' or *D* strands and I types, which are a hybrid between V- and C2-type Ig domains. In contrast to the C-proximal V-type Ig domain, unambiguous assignment of the N-proximal Ig domain of carp SITR as of the V/C2- (or I-) type remains challenging. If our analysis is correct, the organisation of the carp SITR into an N-proximal Ig-like domain of the V/C2- (or I-) type and a C-proximal Ig-like domain of the V-type is unique (5, 6).

To investigate whether the solubility of SITR would be a unique feature of a single gene present in carp only, we used the SITR cDNA sequence to search for orthologues in the genome of zebrafish, a close relative of common carp (59). This search identified 6 sites on 4 different chromosomes (1, 2a, 2b, 15a, 15b and 19) coding for SITR-related molecules (named IGSF), of which several with multiple genes (SUPPLEMENTARY INFORMATION, *see Fig S1*). Carp SITR and SITR-related molecules in zebrafish form a cluster of receptors that all appear to be soluble. However, it is difficult to reliably

predict transmembrane exons from genomic sequences only and thus this prediction will require further research on cDNA sequences from zebrafish. Additional SITR-related zebrafish sequences found on other chromosomal sites do show evidence of transmembrane domains but these sequences form two distinct multigene families. Taken together, these results suggest carp SITR could be an orthologue of a multigenic family of SITRs in zebrafish. If true, the presence of multiple SITRs in carp might help explain the second protein band detected with the anti-SITR antibody.

IgSF structural domain types differ in species distribution. V-types are found throughout all animal species in evolution. C1-types are found only in vertebrates and therefore must have evolved late during metazoan evolution. C2-type Ig domains have been described for *Drosophila melanogaster* and thus are considered to have originated in the protostome lineage. Presently, I-type (and V-type) domains have been found in sub-vertebrate species such as the sea anemone (60). In general, about one-third of the characterized surface proteins of human leukocytes belong to the IgSF. Approximately half of these IgSF proteins contain two Ig domains; an N-proximal V-type followed by a C-proximal C2-type Ig domain (5). In teleost fish, this type of organization can be found in, for example, the NITR family (32, 61). The observed SITR Ig domain organisation may be unusual but is not unique to carp and seems a conserved feature of the SITR-related genes found in the zebrafish genome (unpublished data). The presence of this novel type of IgSF organization may be the result of exon shuffling through intronic recombination as has been described for other members of the IgSF (62, 63). Exon shuffling by which domains can be inserted into a protein, or alternative splicing by which domains can be excluded from a protein, can contribute to addition and deletion of Ig domains from the middle of proteins and therefore give rise to a variety of organisations for IgSF proteins (64). In conclusion, we have identified a protein that may stand model for a novel family of soluble immune-type receptors in fish that displays two unique structural features; (i) the Ig domains are organised as an N-proximal V/C2- (or I) type and a C-proximal V-type and (ii) SITR genes may represent genome-encoded soluble receptors.

The unambiguous assignment of carp SITR sequences as orthologs or paralogs of other presently known immune receptors is difficult at this point. Comparison of structure and chromosomal location of SITRs with previously described Ig-type receptors in zebrafish such as NITRs (31) clearly demonstrates that SITRs form distinct cluster(s) of immune receptor genes. Although alignment and homology searches show a close resemblance of the C-proximal V-type Ig domain of SITRs with human CD300 genes, members of the CD300 family have a single (V-type) Ig domain and usually are transmembrane rather than soluble receptors. CD300 molecules comprise a family of seven members. All members, except for CD300G, possess structural motifs with stimulatory or inhibitory potential (18). Cross-linking CD300 molecules on different leukocyte populations broadly affects gene transcription, phagocytosis, cytokine production, migration and survival (65-67). The cell surface expression of CD300

family members is modulated, in part, by the ability of these molecules to internalize whereas effective CD300 signalling appears to be induced by clustering e.g. into lipid rafts (68). Likely, following the confirmation of carp SITR as a member of a larger family of soluble CD300-related molecules in teleost fish, continued studies on SITRs should provide further insight into the evolution of the CD300 family of molecules.

As predicted from the sequence analysis, SITR protein is not found in the cell membrane but located intracellularly in vesicle-like structures. Upon in vitro stimulation of macrophages with live *T. borreli* parasites SITR protein is readily secreted within minutes. Likewise, we observed a secretion of SITR protein *in vivo* in spleen of *T. borreli*-infected fish already at week 1 post-infection. The predicted secretory nature of SITR would suggest that SITR proteins should be detectable in cell culture supernatants and/or in fish serum. We used western blot analysis with anti-SITR antibody to investigate the presence of SITR in extracellular fluids after *in vitro* and *in vivo* stimulation. Although several templates were tested for the presence of SITR proteins, including: (i) culture supernatants from parasite-stimulated carp macrophages, (ii) parasite stimulated RAW (or HEK) cells overexpressing carp SITR and (iii) serum from *T. borreli*-infected carp, we were unable to detect SITR proteins in solution. A low SITR accumulation rate and/or a short SITR half-life time in the extracellular space likely impair a proper detection of carp SITR in the supernatant. Alternatively, an improved technological approach such as the development of a highly-sensitive monoclonal antibody could optimize the SITR detection in the supernatant.

To assess the function of SITR, we increased SITR protein expression by overexpression in mouse RAW macrophages or reduced SITR protein expression by knock-down in carp macrophages. Cellular activation upon overexpression or inhibition of SITR protein expression was evaluated by means of radical production, phosphorylation analysis and gene expression. Carp SITR itself could act as receptor when overexpressed in mouse macrophage RAW cells and also human HEK cells (unpublished data). Stimulation of these SITR transfectants with live *T. borreli* parasites promoted tyrosine phosphorylation-dependent intracellular signaling cascades. Also, overexpression of SITR in RAW cells increased NO production. The NO induction appeared to be PKC- and partly PI3K-dependent, corroborating the predicted ability of SITR to interact with the PKC kinase and revealing the potential of SITR activation to initiate a phosphorylation-dependent signaling cascade upon stimulation with protozoan parasites. The ability of IgSF receptors to associate with kinases has been proven to exist already in the earliest metazoans such as poriferans (69). Thus, carp SITR (Ig-like domains) proteins may have retained this function. Nitrite production is one of the signature features of *T. borreli* infections of carp as has been shown by strongly increased iNOS gene expression in head-kidney and spleen, increased serum nitrite levels and extensive tyrosine nitration in the spleen (57, 70). *Ex vivo* restimulation of macrophages from *T. borreli*-infected carp with LPS or parasite lysates indicated the presence of classically activated macrophages (caMF) during *T. borreli*

infection (71). Furthermore, NO production during *T. borreli* infection was shown to be protein tyrosine kinase (PTK) and PKC-dependent (58). However, despite all the information acquired on the NO production induced by *T. borreli*, the innate immune receptors implicated in this production are still largely unknown.

Certainly, the increased SITR gene expression in *T. borreli*-stimulated macrophages and the secretion of SITR protein during *in vivo T. borreli* infection suggests a role for this IgSF receptor in the host response to this protozoan parasite. Knock-down of SITR protein expression in carp macrophages confirmed the involvement of carp SITR in the induction of iNOS gene expression by *T. borreli.* A hypothesis could be that an intracellular activating signal from a cell surface-bound receptor, upon recognition of parasite-derived ligand, promotes the interaction of PKC kinase with intracellular SITR and initiate a phosphorylation-dependent cascade leading to NO production. In this situation, extracellular secretion of carp SITR could either represent a strategy to counter-regulate the concentration of intracellular SITR or a strategy to facilitate/antagonize the intracellular SITR-dependent activation (23, 72). Collectively, this study provides a comprehensive analysis, not only *in vitro* but also *in vivo*, of the regulation and putative biological activity of SITR in carp. Further molecular and functional characterization of additional members of an apparent larger family of SITR genes will shed light on the specificity and complementarity of the mechanisms of action between SITR receptors and other innate immune receptors. Finally, we propose a role for carp SITR in the NO-mediated response to the protozoan parasite *Trypanoplasma borreli*.

ACKNOWLEDGMENTS

We thank the central fish facilities, "De Haar-Vissen," for providing and taking care of the carp. Prof. N. Okamoto (Department of Aquatic Biosciences, Tokyo University of Fisheries, Tokyo, Japan) is acknowledged for providing the mouse mAb TCL-BE8. Anja J. Taverne-Thiele, Bas Arkensteijn, Trudi Hermsen and Saskia Rueb (Institute of Biology, Leiden University, Leiden, The Netherlands) are gratefully acknowledged for the technical support.

FUNDING

This study was supported by the Portuguese foundation Fundação para a Ciência e Tecnologia under Grant agreement number SFRH/BD/21522/2005

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SUPPLEMENTARY INFORMATION

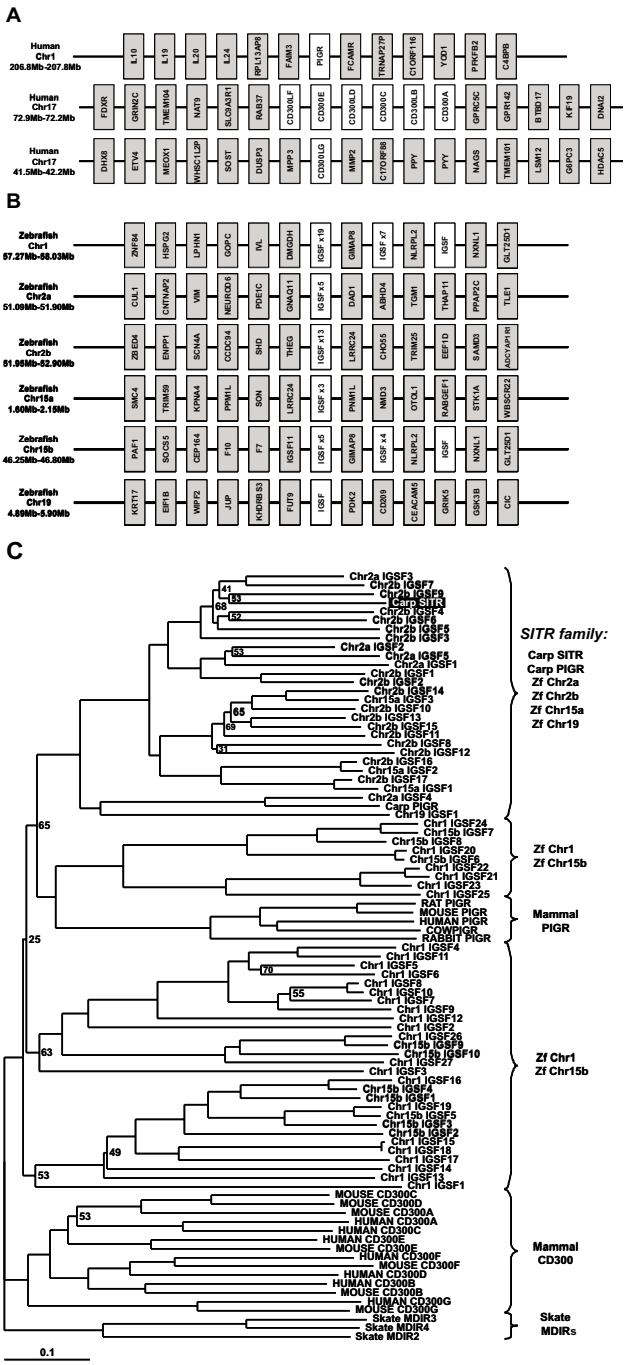
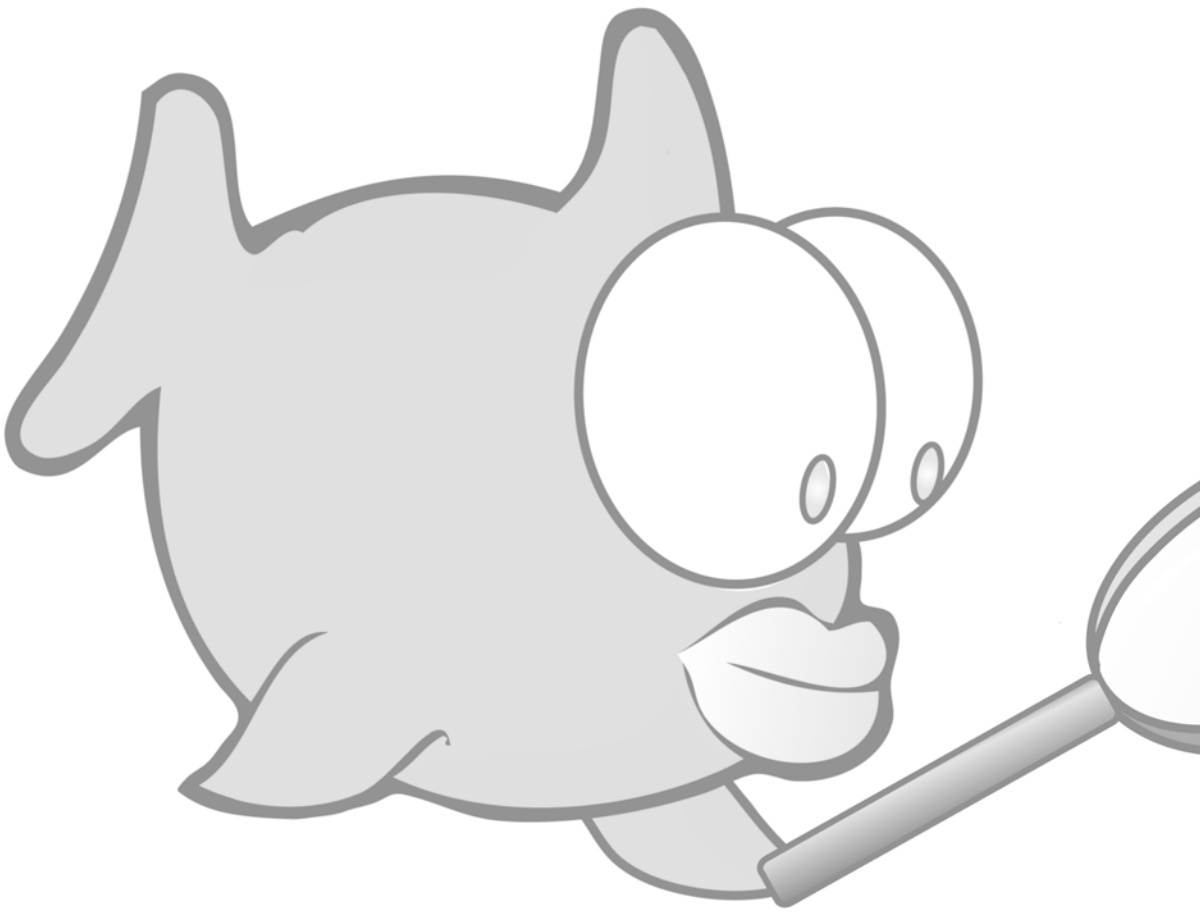


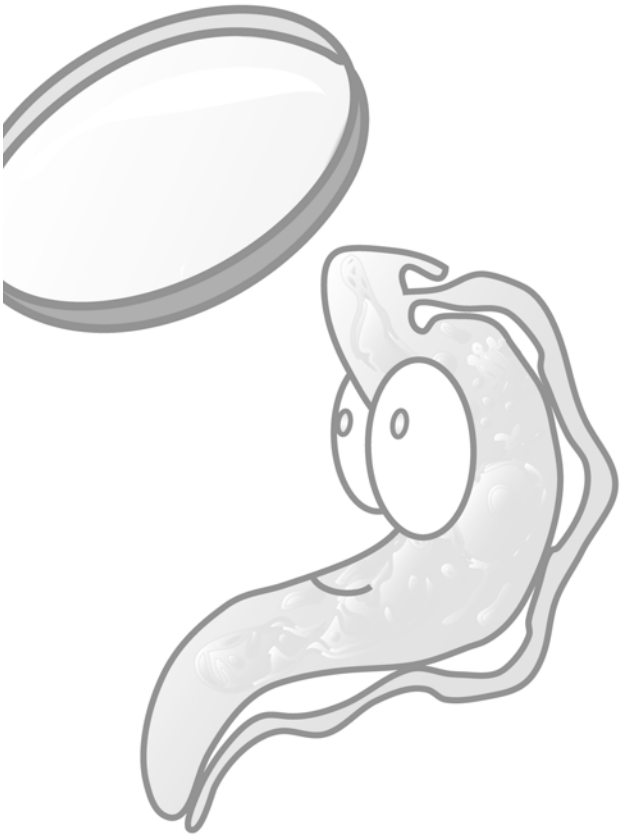
Figure S1: Diversity of carp SITR-related molecules encoded in the zebrafish genome. A. Schematic organization of human CD300 and PIGR loci on chromosomes (Chr) 17 and chromosome 1. B. Schematic organization of zebrafish SITR loci identified on chromosome 1, chromosome 2 (sites 2a and 2b), chromosome 15 (sites 15a and 15b) and chromosome 19. C. Unrooted phylogenetic tree showing the relationship between the carp SITR amino acid sequences for the full-length molecule with other known vertebrate Ig-like receptor sequences. This tree was constructed by the 'neighbour-joining' method using the clustal X and treeview packages, and was bootstrapped 10,000 times. All bootstrap values less than 75% are shown. The GenBank accession numbers (http://www.ncbi.nlm.nih.gov/genbank/) of the human CD300 amino acid sequences are: CD300A, NP_009192.2; ; CD300B, NP_777552.2 ; CD300C, NP_006669.1 ; CD300D, NP_001108624.1; CD300E, NP_852114.1; CD300F, NP_620587.2 ; CD300G, NP_660316.1. The GenBank accession numbers (http://www.ncbi.nlm.nih.gov/genbank/) of the mouse CD300 amino acid sequences are: CD300A, CAM18755.1; CD300B, NP_954691.2 ; CD300C, NP_954695.1; CD300D, NP_663412.1 ; CD300E, NP_742047.1; CD300F, NP_663609.2; CD300G, NP_082263.2. The GenBank accession numbers (http://www.ncbi.nlm.nih.gov/genbank/) of the skate sequences are: MDIR2, ABC86796.1; MDIR3, ABC86797.1; MDIR4, ABC86799.1. The GenBank accession numbers (http://www.ncbi.nlm.nih.gov/genbank/) of the mammalian PIGR are: RAT, NP_036855.1; MOUSE, NP_035212.2; HUMAN, NP_002635.2; COW, NP_776568.1; RABBIT, NP_001164516.1. The Genbank accession numbers (http://www.ncbi.nlm.nih.gov/genbank/) of the carp sequences are: PIGR, ADB97624.1; SITR, HM370297 .



" I think we are blind.
Blind people who can see, but do not see. "

José Saramago

CHAPTER 8



General Discussion

Carla Ribeiro

FISHING FOR TLR LIGANDS

The innate immune system is an ancient evolutionary system of defence against microbial infections that rapidly responds to pathogen-associated molecular patterns (PAMPs). Although much progress has been made towards the identification of innate immune receptors in fish (e.g. this thesis), far less is known about the PAMPs recognized by these receptors. Contaminations of PAMPs with other microbial compounds can make it difficult to unambiguously ascribe activation of particular receptors to the PAMP under study. It therefore is crucial to use highly purified ligand preparations when studying receptor activation and the subsequent biological responses.

For example, involvement of Toll-like receptor 2 (TLR2) in recognition of peptidoglycan (PGN), which is a structural component of the cell wall of almost all bacteria, has been a matter of debate. In mammalian vertebrates, initial experiments with TLR2-transfected HEK 293 cells (1), TLR2-transfected CHO cells (2) and TLR2 knockout mice (3) suggested that PGN is recognized by TLR2. However, a study by Travassos and colleagues (4) challenged this view concluding that PGN may rather be recognized by intracellular Nucleotide Oligomerization Domain (NOD)1 and NOD2 receptors. Subsequent studies by Dziarski and Gupta (5) re-evaluated activation of TLR2 by PGN using two different PGN preparations from *Staphylococcus aureus*: 1) insoluble PGN (iPGN) obtained from cell walls of mechanically-disrupted bacteria, further purified by extraction with SDS or phenol-water, as described previously (6) or 2) soluble PGN (sPGN) purified from supernatants of *S. aureus* grown in the presence of penicillin, which prevents the incorporation of newly synthesized PGN into the bacterial cell wall and therefore results in secretion of sPGN into the medium. As a result of the different treatment, concentrations of more than 10 μg/mL iPGN, in contrast to concentrations of 0.1-1 μg/mL of sPGN were required to activate TLR2-transfected HEK 293 cells. TLR2 activation was abolished by muramidase digestion. The differences in outcome between the above-described studies (recognition of PGN by TLR2 versus NOD) thus could be ascribed to differences in purification procedures. Possibly, TLR2-activating structures of PGN were disrupted by the purification procedure of Travassos (5). No matter what, these studies demonstrate the crucial importance of using highly purified ligand preparations when studying receptor activation, whilst preserving the PAMP-activating structure during the purification procedure.

CANDIDATE TLRS FOR HOST RESISTANCE TO PROTOZOAN INFECTIONS IN CARP

There are several protozoan parasite-derived PAMPs that are candidates for recognition by particular TLRs. Glycosylphosphatidylinositol (GPI) anchors (or their fragments) from *Leishmania major* (7), *Trypanosoma cruzi* (8), *Plasmodium falciparum* (9, 10) and *Toxoplasma gondii* (11) all have been shown to trigger TLR2 and TLR4 activation. In general,

GPI-anchors are composed of a glycan core and a lipid component. Specificity is conferred through variations in the carbohydrate branches, the lipid portion and the number and degree of saturation of the fatty acid chains. Cells of mammalian vertebrates express 10^5 copies of GPI anchors per cell, whereas protozoan parasites express up to 10^7 copies per cell (12). Moreover, protozoan parasite-derived GPI anchors contain a longer glycan core (13). Consequently, both the amount and structure of protozoan parasite-derived GPI-anchors influence the activation of host TLR2 and TLR4.

The surface coat of the protozoan fish parasite *T. carassii*, in particular, is composed of highly glycosylated mucin-like proteins anchored in the plasma membrane by glycosyl phosphatidylinositol (GPI) anchors (14), analogous to the surface coat of *T. cruzi* (15, 16). We made use of the fact that bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) can cleave GPI anchors in eukaryotic cells, and thereby promote the release of soluble proteins containing free GPI-phospholipids and diacylglycerol, to study the possibility that GPI-anchored proteins from *T. borreli* and *T. carassii* could act as PAMPs of carp TLR2.

Recognition of protozoan-parasite derived genomic DNA (containing unmethylated CpG motifs) from protozoan parasites such as *T. cruzi* (17) and *T. brucei* (18) has been shown to be TLR9-mediated. Haemozoin, a product of haemoglobin digested by the intra-erythrocytic protozoan parasite *Plasmodium falciparum* was also shown to be recognized by TLR9 (19). We previously observed that DNase-treatment of *T. borreli* lysates and CpG methylation of *T. borreli* DNA reduced the effectiveness of inducing nitric oxide (NO) in carp macrophages (20). Based on this initial observation, we studied the possibility that *T. borreli* DNA could contain PAMPs of carp TLR9.

Carp TLR2 ligands

At the beginning of our studies, the TLR2 gene had been identified in a number of teleost fish species but these studies had been mostly restricted to sequence identification and determination of basal and inducible levels of gene transcription (21-24). However, activation of fish cells by a prototypical TLR ligand provide no evidence for a direct relationship between the ligand, the corresponding receptor and the resulting immune response and may lead to false presumptions on functional conservation (25). We cloned the carp TLR2 gene and investigated the role of the TLR2 receptor in recognition of ligands from Gram-positive bacteria and from protozoan fish parasites.

In mammalian vertebrates, HEK 293 cells are widely used to study receptor activation, often measured via NF-κB activation. HEK 293 cells have no or negligible native TLR expression and thus represent a valuable tool to study TLR activation. Transfection of HEK 293 cells with carp TLR2 confirmed the ability of prototypical TLR2 ligands (LTA, PGN and Pam_3CSK_4), but not the mammalian TLR2 ligand MALP-2, to trigger MAPK-p38 phosphorylation and therefore confirmed recognition by carp TLR2. Overexpression of TLR2 in carp macrophages corroborated reactivity of carp TLR2 to LTA, PGN and Pam_3CSK_4

(26). We applied the same approach to study protozoan fish parasite-derived ligands for carp TLR2. Both transfection of HEK 293 cells and overexpression of TLR2 in carp macrophages showed the ability of live *T. carassii* parasites and GPI-anchors from *T. carassii* parasites, more than *T. borreli*-derived PAMPs, to be recognized by carp TLR2 and trigger MAPK-p38 phosphorylation. Our results suggest a conservation of the binding ability to Gram-positive derived ligands (LTA and PGN) and protozoan parasite-derived ligands (GPI-anchors) for carp TLR2. Conservation of TLR2 ligand binding specificity proposes an essential role for TLR2 in the recognition of these two groups of microorganisms throughout evolution.

TLR2 FUNCTION: DIFFERENTIAL ACTIVATION BY LIGANDS FROM GRAM-POSITIVE BACTERIA

In our studies, LTA consistently stimulated downstream cytokine gene expression with a delayed kinetics when compared with PGN. Differences in kinetics have been suggested to be the result of distinct TLR2 heterodimer and co-receptor (CD14, CD36, RP105,TLR1 and TLR6) usage and signalling after cellular trafficking of these complexes (27-32). Additionally, histone modification and increased promoter access by distinct microbial products can promote transcriptional specificity and help to establish unique transcriptional responses downstream of distinct TLRs (33). In addition, PGN always more clearly than LTA induced NO, ROS production and MAPK-p38 phosphorylation (26). Thus, we observed ligand-specific cellular responses to distinct carp TLR2 ligands suggesting that activation of TLR2 can promote different outcomes depending on the ligand.

In mammalian vertebrates, TLR2 activation by LTA induces unique inflammatory responses when compared to other TLR2 ligands (32). Also, TLR2 can respond differently to PGN from Gram-positive (lysine-containing) and Gram-negative (diaminopimelic (DAP)-containing) bacteria (34). Gram-positive bacteria have a very thick lysine-containing PGN layer whereas Gram-negative bacteria contain only a thin DAP-containing PGN (35). With respect to Nucleotide Oligomerization Domain (NOD) proteins, two different receptors have evolved that either respond to DAP- (NOD1) or lysine- (NOD2) containing PGN (36, 37). In the case of TLR2, the single receptor can recognize PGN from both Gram-positive as well as Gram-negative bacteria. Yet, TLR2 responds differently to these two classes of compounds due to different binding modes of lysine- and DAP-containing PGN (34). A wider range of DAP-containing PGN bound with higher affinity to TLR2 than lysine-containing PGN. It has been proposed that the difference in recognition of the two classes of PGN is a host strategy to respond appropriately to Gram-negative and Gram-positive bacteria, which produce vastly different quantities of PGN. Thus, TLR2 may have evolved to recognize a limited number of Gram-positive PGN motifs to avoid over-activation of innate immune responses (34). Teleost fish are relatively hyporesponsive to lipopolysaccharide (LPS) from Gram-negative stimulation and fish TLR4 does not recognize LPS (25, 38, 39). Alternatively, recognition of Gram-negative PGN, possibly in a TLR2-dependent manner, could represent an important strategy to also recognize Gram-negative bacteria in fish.

TLR2 function: polarization during protozoan parasite infection

In addition to direct activation of innate host-defence mechanisms, PRRs can have the ability to shape the outcome of adaptive immune responses. PRR-induced signals, including cytokines and cell-surface-associated molecules, result in Th-cell activation and differentiation into one of the several types of effector Th-cells characterized by the secretion of (sets of) signature cytokines (40, 41). Activation of TLR can lead to synthesis of different IL-12 cytokine family members (42-44). For example, in mammalian vertebrates, TLR9 activation (by CpG oligodeoxynucleotides) strongly induces IL-12 production whereas TLR2 activation (by PGN) preferentially leads to IL-23 production. IL-12 is a key factor for the Th 1 cell differentiation whereas IL-23 contributes to the expansion of Th17 cells. Th 1 cells produce IFN-γ and activate macrophages to produce inflammatory mediators crucial in the defence against intracellular pathogens. Th 17 cells produce IL-17 which induces the production of chemokines important for the recruitment of neutrophilic granulocytes to the site of infection (45-47). We characterized the type of immune response (Th 1 versus Th 17) based on cytokine gene expression and characterization of associated cellular responses.

T. borreli infections in carp are associated with production of $NO^{\cdot}$, TNF-α and IFN-γ (48-50) whereas *T. carassii* infections seem to inhibit $NO^{\cdot}$ production and last 2-3 weeks longer (48, 51, 52). In our study, protozoan parasite *T. carassii*- more than *T. borreli*-derived PAMPs stimulated TLR2-induced MAPK-p38 activation. Moreover, overexpression of TLR2 in carp macrophages established a TLR2-mediated up-regulation of p19 by *T. carassii* but not by *T. borreli* parasites. Thereby, our data provide a link between activation of TLR2 by *T. carassii* parasites and downstream induction of p19 gene expression, as a measure for IL-23 production. During *T. borreli* infection of carp we observed a moderate increase in the number of splenic macrophages and a strong induction of IFN-γ2 in head-kidney and spleen. In contrast, a strong neutrophilia in the spleen of *T. carassii*-infected fish, induction of chemokines and induction of IL-17A/F2 gene expression were observed during *T. carassii*-infection. These observations are in agreement with previous reports that associate *T. borreli* infection with the development of a Th1-like immune response and provide evidence of the development a Th17-like immune response during *T. carassii* infections of carp (Fig.1). Furthermore, our date suggest that TLR2 is involved in polarization of the immune response towards a Th17-like phenotype during *T. carassii* infection.

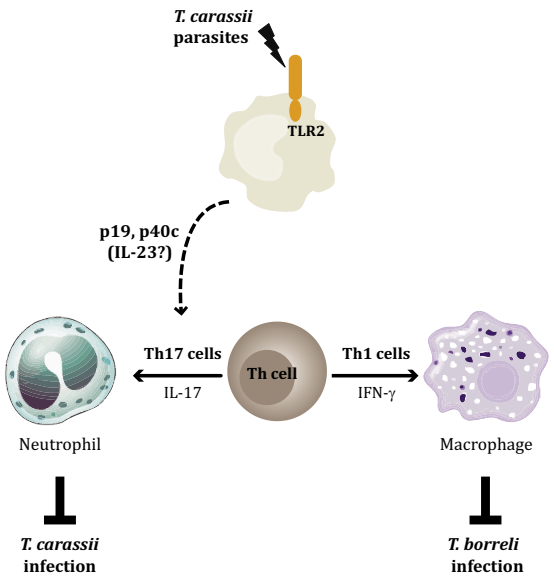


Figure 1. Schematic representation of the polarization of the immune response to protozoan parasites infections in carp. Th cells can differentiate into, among others, Th1 and Th17 effector cells depending on the infecting pathogen. Each Th subset is characterized by the set of cytokines they produce and the immune effector mechanisms induced. It is possible, but not proven (denoted by dotted arrow), that the preferential TLR2-induced p19 and p40c gene expression lead to a functional IL-23 protein and promote Th17-like responses in *T. carassii* infections. *T. borreli* infections are associated with Th1-like responses which are apparently not IL-12-driven. Adapted from (41).

MANIPULATION OF TLR-MEDIATED RESPONSES BY PROTOZOAN PARASITES

TLR-initiated NF-κB and MAPK transduction cascades are evolutionary ancient signalling pathways that culminate in the induction of multiple proinflammatory genes that are important in defense against protozoan infections. These proinflammatory genes include IL-12, TNF-α and iNOS. Several lines of evidence demonstrate the ability of protozoa to modulate signalling pathways and consequently inhibit IL-12 production (44, 53).

Leishmania promastigotes have been shown to inhibit IL-12 production, this effect being mediated by cell surface lipophosphoglycan (LPG) targeting MAPK-ERK (54). Furthermore, *Leishmania* amastigotes, but not promastigotes, are able to disrupt NF-κB activation leading to an impaired IL-12 production by macrophages. The proteolytic breakdown of host NF-κB and IκB is mediated by amastigote-specific cysteine peptidase (55). A recent study demonstrated that *Leishmania*-induced IL-12p40 suppression is transcriptionally-mediated, requires cell signalling and phagocytosis. Moreover, infection with *Leishmania* suppresses IFN-γ- and LPS-induced IL-12p40 production (56). Of note, GPI anchors as well as LPG are TLR2 ligands. In contrast, *T. cruzi* trypomastigotes express GPI mucin that induces potent IL-12 production, whereas LPG and synthethic Pam_3CSK_4

have been reported to inhibit IL-12 production (57). Therefore, not all TLR2 ligands from protozoan parasites act to downregulate IL-12 production directly. Following infection with *Toxoplasma gondii*, macrophages, neutrophilic granulocytes and dendritic cells become unresponsive to LPS-induced activation as measured by the production of IL-12 and TNF-α (58, 59). *T. gondii* interferes with LPS-induced MAPK-p38, which is required for IL-12 production (60). It has been suggested that MAPK-p38 inactivation by *T. gondii* results from deactivation of upstream p38-activating kinase or from induction of a MAPK-p38 phosphatase which prevents phosphorylation and therefore activation of this kinase by *T. gondii*. A recent report observed an inhibition of IL-12 production by the extracellular blood parasite *T. brucei gambiense* in rat macrophages. M-CSF and NO increased during infection, however M-CSF only appeared to be implicated in the IL-12 suppression by *T. brucei gambiense* (61).

We observed that both *T. borreli* and *T. carassii* extracellular blood parasites of carp interfere with PGN-induced IL-12 gene transcription. p35 gene expression was suppressed during infection with both protozoan parasites. Similarly, we observed a selective suppression of p40a during *T. borreli* infection and a very low induction of p40a gene expression during *T. carassii* infection. Despite the apparent IL-12 deficiency during these protozoan infections of carp, IFNγ2 gene expression was enhanced. Studies in mammalian vertebrates indicate a role for endogenous host mediators produced during infection as being responsible for IL-12 suppression. The anti-inflammatory cytokine IL-10, which is induced during infection with protozoan infection, is well known for its ability to downregulate pro-inflammatory mediators such as IL-12 or TNF-α. In addition, TNF-α itself has been shown to selective inhibit p40 transcription and IL-12 (IL-12p70) secretion induced by IFN-γ2 and *S. aureus* in human macrophages (62). The mechanisms by which protozoan parasites suppress IL-12 production in carp during infection remain largely unknown.

T. borreli infections of carp are characterized by an increase of TNF-α gene expression already at the beginning of infection (49) and high levels of NO (20), ROS (63) and IFN-γ2 (50) gene expression at the peak of parasitaemia, associated with a type-1 immune response. Differently, *T. carassii* infections of carp are characterized by an inhibition of NO production during the whole infection period (51) and an increase of IFN-γ2 and IL-17 A/F2 gene expression, associated with a mixed type-1/17 immune response. Our data suggest that protozoan derived-PAMPs, in particular *T. carassii*-derived PAMPs, are able to promote TLR2-induced MAPK-p38 activation in HEK 293 cells. Moreover we followed the basal and H_2O_2-induced MAPK-p38 activation during *T. borreli* and *T. carassii* infections. We observed a suppression of MAPK-p38 induction at week 1 and 5 of both parasites. At the peak of parasitaemia (week 3) we observed a moderate increase of the basal MAPK-p38 induction and a striking increase of H_2O_2-induced MAPK-p38 activation. We also observed a TLR2-induced MAPK-ERK suppression in HEK 293 cells by both live *T. borreli* and live *T. carassii* parasites (unpublished data). These observations suggest that both protozoan parasites interfere with MAPK-dependent cascades downstream of TLR2

activation. The temporal *in vivo* inactivation of MAPK-p38 or the *in vitro* ability to suppress TLR2-induced MAPK-pERK by both protozoan parasites could contribute to the observed suppression of IL-12 production during infections of carp with the protozoan parasites *T. borreli* and *T. carassii*.

CARP TLR9 LIGANDS

At the beginning of our studies, the TLR9 gene had been identified in a number of teleost fish species, but these studies had been mostly restricted to sequence identification and determination of basal and inducible levels of gene transcription (64-67). We cloned the carp TLR9 gene and investigated the role of the TLR9 receptor in recognition of DNA ligands.

In our studies, we initially used unmethylated-CpG containing DNA as prototypical ligands to study activation of carp TLR9. HEK 293 cells were transfected with carp TLR9 and MAPK-38 phosphorylation was used as measure of responsiveness for TLR9 activation. However, none of the CpG motifs analysed, although previously shown to be stimulatory in fish and/or mammals, were strong inducers of carp TLR9 activity (68, 69). These results underscore the well-known species-specific CpG binding structure specificity of TLR9 (70) and suggest the optimal stimulatory CpG motifs for carp TLR9 have yet to be defined.

We used as sources for pathogen-derived (unmethylated-CpG rich) DNA both bacterial (*E. coli*) and protozoan parasite (*T. borreli*) DNA to study recognition by carp TLR9. We used bacterial *E. coli* DNA complexed with a lipid-based transfection reagent as source for bacterial DNA. Stimulation of carp macrophages with this complexed bacterial DNA resulted in much stronger cellular activation than uncomplexed *E. coli* DNA. Possibly, complexed trypanosome DNA would be more efficiently recognized by carp TLR9. Moreover, the degree of cytosine methylation for eukaryotic protozoan DNA is expected to be higher than the degree of cytosine methylation found in prokaryotic bacterial DNA (71). These differences in degree of methylation could explain why bacterial DNA, but not protozoan parasite-derived DNA, could be proven a carp TLR9 ligand.

TLR9 FUNCTION: PROTEOLYTIC REGULATION

In addition to the induction of distinct signaling pathways, TLRs sample different compartments within cells. TLRs involved in the recognition of nucleic acids (TLR3, TLR7, TLR8 and TLR9) are localized within the endolysosomal compartment, whereas other TLR family members (TLR1, 2, 4 and 6) are found at the cell surface (72). In mammalian vertebrates, the cellular localization of TLR9 has important consequences for ligand accessibility and downstream signaling events (73, 74). Internalization of exogenous DNA induces the translocation of TLR9 from the ER, through the Golgi, to the DNA-containing endolysosomes. In the endolysosomes, resident proteases cleave the TLR9 at the flexible

loop site (at LRR15) leading to the generation of a cleaved form of the TLR9 receptor (C-terminal fragment starting from LRR16) (75, 76). Controlled proteolysis thus seems important for the function of TLR9.

The presence of a flexible loop in the carp TLR9 molecule and the protease-dependent TLR9 activation in carp macrophages suggest an evolutionary conservation of the proteolytic regulation of TLR9 activation. First, the three-dimensional model of carp TLR9 confirmed the presence of a flexible loop at LRR15. This flexible loop for TLR9 was predicted to be longer in several fish species than in mammalian vertebrates. At this moment it is unknown if the length of the flexible loop can influence the function of fish TLR9. Second, IL-1β and p35 gene expression, induced by the prototypical TLR9 ligand *E. coli* DNA, was protease-dependent in carp macrophages.

In mammalian vertebrates, both full-length and truncated forms of TLR9 can bind ligand (75, 76). However, MyD88 was shown to be selectively recruited to the truncated form of TLR9 but not to the full-length receptor (75). A recent report indicated that pre-assembled TLR9 homodimers undergo ligand-induced conformational changes enabling MyD88 recruitment (77). It is possible that truncation of the TLR9 ectodomain increases ligand-binding affinity or that truncation allows the TLR9 to adopt important structural changes required for signal initiation (74). However, a role for only the truncated form in activation of the TLR9 receptor seems in contrast with the role for the leucine-rich repeat (LRR) regions LRR2, 5 and 8 (N-terminal fragment) of TLR9 for ligand binding (78). In conclusion, the recent data on mammalian vertebrate TLR9 suggest that the full-length and truncated forms of TLR9 display different ligand-binding sites and may play distinct roles in TLR9-dependent signal activation or regulation. At this moment, the exact functional consequences of TLR9 cleavage for ligand binding and immune responsiveness are unknown.

The presence of a flexible loop in carp TLR9 may not only render it susceptible to proteolysis by host proteases resident in endolysosomes, but may also render TLR susceptible to proteases from pathogens acquired after phagocytosis of pathogens. For example, cathepsin L-like proteases of trypanosomes are thought to contribute not only to pathogenesis (e.g. by digestion of hemoglobin) but also to modulation of the host immune response (e.g. by degradation of IκB, NF-κB) (79, 80). We have cloned, characterized and produced a recombinant cathepsin L-like cysteine protease from *T. borreli* and have shown this protease to be biologically active and capable of digesting carp (host) haemoglobin, immunoglobulin and transferrin (81). It would be interesting to study the possibility that *T. borreli* cysteine protease could be capable of digesting host TLR9 and thereby modulate the function of carp TLR9.

Fish possess a TLR9 homologue which responds to DNA from pathogens, similar to the situation in mammalian vertebrates. Interestingly, fish also possess TLR21, the receptor for CpG DNA in chicken and apparently replacing the TLR9 receptor that is missing from the chicken genome (82). Chicken TLR21, however, has structural and functional characteristics different from TLR9 of mammalian vertebrates (83). For example, chicken

TLR21 does not have the flexible loop present on LRR15 characteristic of TLR9, suggesting TLR21 may not be protease-dependent for activation. No matter what, the presence in the genome of fish (at least zebrafish and pufferfish) and also the genome of frogs, of two TLR receptors (TLR9 and TLR21) that may recognize DNA is remarkable (84). In this context it may be particularly rewarding to study the function of a group of TLRs that are specifically found in fish and amphibians; TLR21, 22 and 23. In pufferfish there are two TLR receptors that recognize RNA; TLR22 responds to long-sized dsRNA whereas TLR3 responds to short-sized dsRNA (85). Possibly, aquatic animals have also developed two TLR receptors (TLR9, TLR21) that can recognize DNA, but this remains an issue for future research.

SOLUBLE RECEPTORS: GENERATION AND FUNCTION

Proteolytic cleavage of receptor ectodomains is one of the mechanisms used to generate soluble receptors. Alternatively, soluble receptors can be generated by alternative splicing of mRNA transcripts, release of full-length receptors within the context of exosome-like vesicles, cleavage of GPI-anchored receptors but also by transcription of distinct genes that encode soluble receptors, such as seems to be the case for fish SITRs. Soluble receptors can act as (i) nonsignalling decoy receptors, (ii) receptor-associated proteins, (iii) soluble receptor antagonists or (iv) signalling receptors (Fig.2).
(i) One model of soluble receptor function is the removal of the receptor from the cell surface so that it can no longer serve as a signalling molecule. (ii) In another model of soluble receptor function, the soluble receptor is a binding protein that protects its ligand from degradation or protein clearance in the extracellular space. (iii) The most familiar notion of soluble receptor action is as antagonists of their membrane-bound counterparts. (iv) In addition to inhibiting the effects of their cognate ligands, soluble receptors can also facilitate ligand-mediated signalling (86-88).

LAIR-2, sTREM-1 and sTLT-1 are examples of Ig-like soluble receptors described in mammals. Human LAIR-2 is a soluble, high affinity collagen receptor which prevents binding of the membrane-bound human LAIR-1 to collagens behaving, therefore, as a soluble receptor antagonist (89). Studies in mouse models have implicated the soluble form of TREM-1 (sTREM-1) in innate immune response to infection, sepsis and granulomatous disease. Stimulation of mouse monocytes with TLR ligands promoted the release of sTREM-1 into the culture supernatant and sTREM-1 has been detected in plasma during experimental and clinical sepsis (90-92). A soluble form of TLT-1 (sTLT-1) is released by activated platelets and can be detected in human serum. During inflammation, soluble sTLT-1 can act as a signalling receptor and functions to augment actin polymerization in platelets leading to increased aggregation and adherence to the endothelium (93-95). No matter what action these soluble receptors have, their function usually is combined with functions of membrane-bound receptors of the same family.

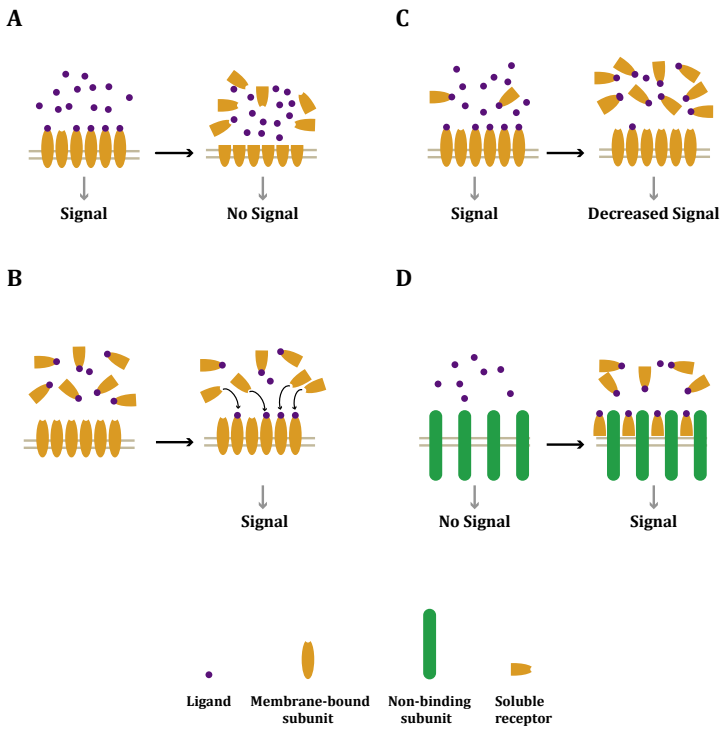


Figure 2. Mechanisms of action of soluble receptors. A) nonsignalling decoy receptors (i), B) receptor-associated proteins (ii), C) soluble receptor antagonists (iii) or D) signalling receptors (iv). Adapted from (86).

In our study, the soluble immune-type receptor (SITR) is a soluble receptor encoded in the genome and does not seem to have membrane-bound family members. It therefore is difficult to assign a function to carp SITR based on the mechanisms of action described for soluble receptors that do have membrane counterparts, as described above. Nevertheless, knock-down of SITR protein expression using morpholino antisense technology and overexpression of SITR in mouse RAW macrophages provided evidence for a role of SITR in the NO-mediated response of carp to *T. borreli* infection. We hypothesize that a *T. borreli*-induced signal via a putative surface receptor could promote the interaction of SITR with PKC and therefore be involved in the NO-mediated response. Secretion of SITR to the extracellular space could represent a strategy to counter-balance the intracellular SITR protein expression. Future studies aiming at characterization of the fate and function of secreted SITR should help define in more detail SITR function.

SITR REPERTOIRES IN TELEOST FISH: THE MORE THE BETTER?

The primary advantage of innate function is to provide immediate recognition of the pathogen and a temporal buffer for the development of an adaptive immune response. Long-term coexistence between animals and microorganisms might have favoured the evolution of large families of innate immune receptors for microbial recognition molecules. The availability of genome information for zebrafish enabled us to search for SITR orthologues by synteny (96). We identified in zebrafish the presence of a large multigenic SITR family. Comparison of structure and chromosomal location of SITRs with previously described Ig-type receptors in zebrafish such as NITRs clearly demonstrates that SITRs form distinct cluster(s) in the genome (97). True for all jawed vertebrates, the extensive diversification of antigen-binding receptors depends on somatic gene rearrangement (98, 99). The high degree of germline variation in innate receptors (e.g. TLRs) is one of the alternative mechanisms used by jawless vertebrates such as sea urchin and amphioxus to create diversification (100-102). Similarly, an expanded immune receptor repertoire in fish might contribute to a higher diversity of immune recognition capacities by spatiotemporal regulation of the SITR repertoire. Therefore, it is conceivable that a large SITR protein repertoire with distinct protein expression patterns and multiple combinatorial possibilities would exist. This repertoire would have the potential to recognize a multitude of diverse ligands and fine-tune immune responses. The unexpected number of multigenic IgSFs and mechanism of IgSF receptor diversification identified in various jawless vertebrates (84) underscores the possibility that alternative mechanisms of immunity are yet to be discovered. Molecular characterization of other members of the SITR family will help elucidate the cooperative interactions and functional redundancy among the SITR family and other innate immune receptors in fish.

AN INTEGRATED VIEW AND OUTLOOK

The recognition of pathogens is mediated by several families of innate immune receptors that collectively sample the extracellular space, endolysosomal compartment and the cytoplasm for signs of infection or tissue damage. Both TLR2 and TLR9 are membrane proteins, but expressed in distinct cellular compartments. TLR2 is expressed in the plasma membrane whereas TLR9 is expressed in intracellular compartments and mainly active in the endolysome membrane of carp macrophages. SITR, however, is a soluble receptor expressed abundantly in the cytoplasm of carp macrophages and apparently contained in vesicle-like structures. We defined as ligands for carp TLR2 GPI-anchors from protozoan parasites and LTA and PGN from Gram-positive bacteria. Carp TLR2 therefore has, similar to mammalian vertebrate TLR2, a broad specificity. On the one hand, we observed ligand-specific kinetic profiles and ligand-specific downstream cytokine induction upon activation of carp TLR2. On the other hand, preferential TLR2-induced p19 gene expression during *T. carassii* infection

underlines the potency of TLR2 to polarize immune responses towards a Th17-like phenotype. In contrast, *T. borreli*–derived PAMPS were less stimulative for TLR2 and are associated with the development of Th1-like immune responses. For future experiments, it would be interesting to study the degree to which the broad specificity and differential activation of carp TLR2 could be the result of heterodimerization with other receptors (e.g. TLR1(103)) or cooperative signalling interaction with other receptors (e.g.TLR4, TLR9 (104)). We have recently identified TLR1 (*I. Fink, personal communication*) and TLR4 (*D. Pietretti, personal communication*) in carp. Co-transfection studies with carp TLR2 and knock-down studies using morpholinos designed against each of these TLRs will help to further delineate the TLR2 network in carp.

Although the molecular structure recognized by carp SITR is as yet unidentified, our results implicate the involvement of carp SITR in the host response to infection with *T. borreli*. Both, the overexpression of SITR in RAW macrophages and the knock-down of SITR in carp macrophages provided evidence for involvement in the NO-mediated response against *T. borreli*. We propose that interaction of SITR with the kinase PKC could lead to a subsequent tyrosine phosphorylation cascade culminating in the induction of iNOS gene expression and therefore NO production. However, the signaling characteristics resulting in intracellular SITR activation and the fate and function of secreted SITR are some of the issues that remain largely unknown (Fig. 3). Immunoprecipitation of (tagged) carp SITR from supernatants collected from SITR-transfected cells and in vitro stimulation of carp macrophages or other cell types with secreted recombinant SITR protein might help to identify the signalling abilities of secreted SITR molecules. In addition, because the production of NO seems to be inhibited during *T. carassii* infections, it would also be interesting to study the outcome of *T. carassii* stimulations of carp macrophages following a previous knock-down of SITR. These experiments would help further characterize the function of carp SITR. Moreover, initial screening aiming at defining the basal and inducible gene expression patterns of each of the zebrafish SITR members in adult zebrafish could help to scrutinize the members that could play a major role in the immune response. After confirming the expression of SITR in zebrafish embryos, morpholinos directed at each member could help to delineate the role during infection such as *Salmonella typhimurium* (105, 106) and *Mycobacterium marinum* (107, 108) in zebrafish. These experiments would help to characterize the function of zebrafish SITR family members.

The subtracted cDNA library also identified two C-type lectin receptors, interferon-regulatory factor 7 (IRF-7), NF-κBε and cystatin as enriched transcripts in carp macrophages stimulated with live *T. borreli* parasites. We have recently cloned the full-length sequences of these two C-type lectin receptors and they appear to be soluble receptors (*A. Oostegaard, personal communication*), similar to carp SITR. This raises the question whether these C-type lectin receptors could interact with members of the SITR family. Both IRF-7 and NF-κBε are well-known for their involvement in TLR9 signalling cascades and cystatin is a cysteine protease inhibitor. This suggests that TLR9 may, in fact, be involved in the recognition of *T. borreli* derived PAMPs and that inhibition of *T. borreli* cysteine protease by cystatin might

be a strategy to hamper modulation of host proteins (e.g. TLR9) by the parasitic cysteine protease. Future use of a more refined method (e.g. parasite DNA complexed with lipid based transfection) to increase the uptake of protozoan DNA by carp macrophages might result in activation of TLR9. Stimulation of carp macrophages and TLR9-transfected HEK 293 cells with *T. borreli* cysteine protease could then help unravel a putative role for this parasitic cysteine protease in regulation of TLR9 activation.

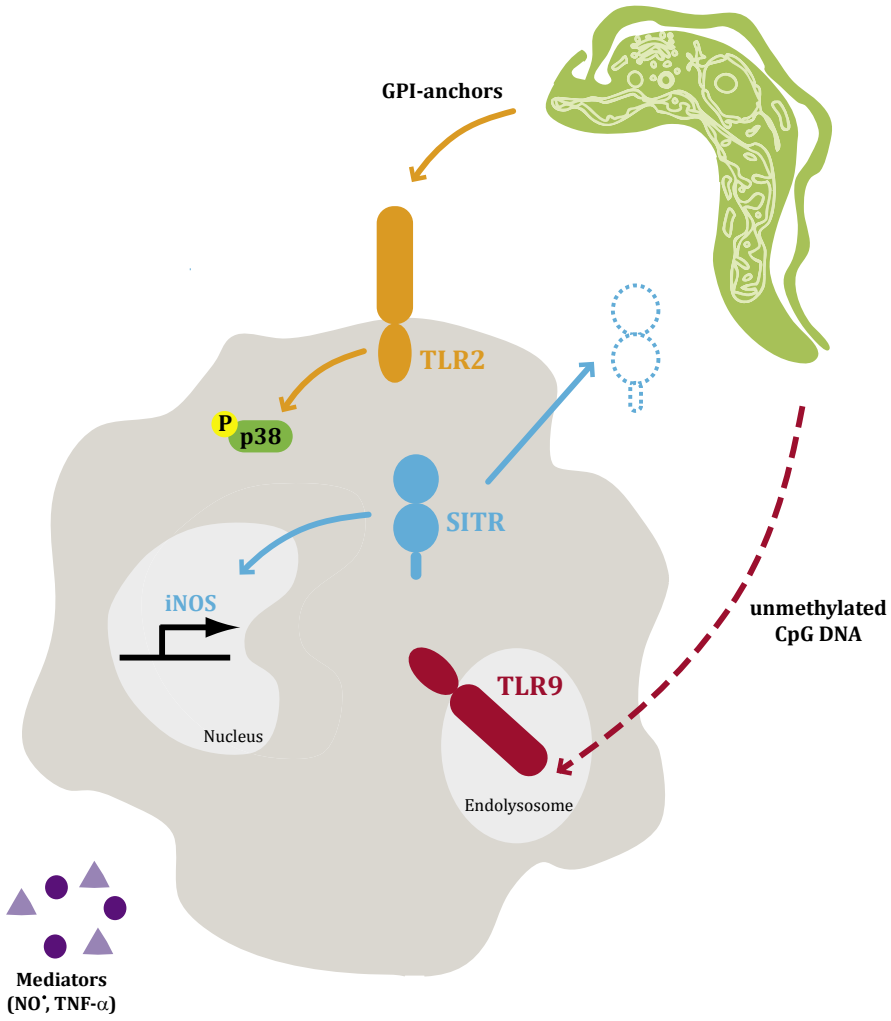


Figure 3. Overview of the innate immune receptors in carp macrophages involved in the recognition of protozoan parasite *T. borreli*. *T. borreli* infections are characterized by the production of mediators such as nitric oxide ($NO^{\cdot}$) (48) and Tumor necrosis factor-α (TNF-α) (49). TLR2 is able to recognize GPI-anchors from *T. borreli* and initiate MAPK-p38 activation. TLR9 activation by unmethylated CpG DNA from *T. borreli* parasites remains to be elucidated. Carp SITR is secreted upon *T. borreli* stimulation, however, we were unable to detect the secreted SITR. Carp SITR is involved in the iNOS gene expression induced by *T. borreli* stimulation.

Conclusions

In this thesis we aimed at the identification of innate immune receptors involved in the recognition of the protozoan parasites *T. borreli* and *T. carassii* that infect carp. TLR2 and TLR9 were selected as candidate receptors based on literature studies of host-parasite interactions in mammalian vertebrates. Carp SITR was found by subtractive suppression hybridization, in a cDNA pool enriched for genes up-regulated in response to the protozoan parasite *T. borreli*. We established the ability of carp TLR2 and of carp SITR to respond to protozoan parasite-derived ligands. The implication of carp TLR9 in the recognition of nucleic acids from protozoan parasites remains elusive. Immune responses of carp to *T. borreli* and *T. carassii* are fundamentally different. We propose that a preferential TLR2-regulated Th17-like immune response is induced by *T. carassii* parasites and provide evidence for the involvement of carp SITR in the NO-mediated immune response to *T. borreli* parasites.

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Summary

Samenvatting

Sumário

Summary

Adaptation and evasion strategies have allowed parasites of the Order Kinetoplastida to persist in almost all vertebrate groups and infections with these protozoan parasites are widespread not only among warm-blooded but also among cold-blooded vertebrates. In **CHAPTER 1**, we introduced the hypothesis pertinent to this thesis that carp (*Cyprinus carpio* L.) macrophages bear pattern recognition receptors (PRRs) essential to the recognition of the protozoan parasites *Trypanoplasma borreli* or *Trypanosoma carassii* and central to the development of innate immune responses.

Once a pathogen has breached physical barriers it can be recognized by PRRs on cells of the innate immune system, triggering a whole series of immune responses aimed at removal of the pathogen. PRRs, therefore, play a central role in the detection of molecular structures unique to pathogens (providing what is called 'signal 0'). Macrophages, among others, can present pathogen-derived antigen to cells of the adaptive immune system (signal 1) and secrete cytokines that shape the adaptive immune system (signal 2) leading to pathogen clearance. The two latter signals are both required for activation of specific B and T lymphocytes. Adjuvants are categorized as "signal 1 facilitators" and /or "signal 2 facilitators" (**CHAPTER 2**) and by these mode of actions can lead to a more efficient design of vaccines, also for aquaculture practice.

To study the functional consequences of innate immune receptor engagement, we first developed an *in vitro* culture system to obtain relatively pure cultures of macrophages derived from the head-kidney of carp. These macrophages, best compared to bone marrow-derived macrophages, retained the ability to phagocytose and produce radicals (**CHAPTER 3**), and were central to our further studies on the recognition of PRRs from protozoan parasites. Among the several distinct classes of PRRs able to recognize a large array of pathogen associated molecular patterns (PAMPs), without doubt, the family of Toll-like receptors (TLRs) are a very important class of PRR.

We first investigated, in detail, the role of the carp TLR2 receptor in the recognition of prototypical ligands. Transfection of human HEK 293 cells with TLR2 corroborated the ability of carp TLR2 to bind LTA and PGN from *Staphylococcus aureus* and the synthethic triacylated lipopeptide Pam_3CSK_4, but not the diacylated lipopeptide MALP-2 (**CHAPTER 4**). The use of the same ligands on carp macrophages overexpressing TLR2 indicated that carp macrophages require high concentrations of LTA and PGN, react less strongly (Pam_3CSK_4) or do not react at all (MALP-2), thus showing a difference in immune response of the TLR2-containing receptor complex between mammalian vertebrates and carp.

Live protozoan parasites *T. borreli* or *T. carassii* and GPI anchors derived from both protozoan parasites were examined as TLR2 ligand. During an immune response, the balance between interleukin (IL)-12 (p35, p40) and IL-23 (p19, p40) can depend on the nature of the PAMP recognized by TLR2. Transfection studies of HEK 293 and carp macrophages showed that *T. carassii*-derived PAMPs are agonists of carp TLR2, promoting p19 and p40c gene expression (**CHAPTER 5**). During *T. carassii* infections, in particular, we observed a propensity to induce p19 and p40c gene expression, suggestive of the formation of IL-23. IL-17A/F2 gene expression was observed only during *T. carassii* infections. We

could detect a marked increase in the number of splenic neutrophilic granulocytes during *T. carassii* infection, along with an increased gene expression of metalloproteinase-9 and chemokines, providing evidence for a Th17-like immune response in carp in response to infection with *T. carasssii*.

TLR9 is an important PRR that senses, among others, DNA-containing unmethylated CpG motifs found in bacterial and protozoan (trypanosome) DNA. Carp TLR9 was shown to have a TLR7 family signature with 26 leucine-rich repeats (LRR) and an extended flexible loop in LRR15 (**CHAPTER 6**). Carp TLR9, when transfected into human HEK 293 cells, recognized *E. coli* DNA, but not protozoan *T. borreli* DNA or any of the tested CpG motifs. Inhibition of protease activity in carp macrophages decreased cytokine gene expression following stimulation with *E. coli* DNA, suggesting the presence of protease-dependent TLR9 activation routes for carp TLR9.

Investigation of a substracted cDNA repertoire from carp macrophages enriched for genes up-regulated in response to *T. borreli,* identified a novel soluble immunoglobulin-like receptor which we named Soluble Immune-Type Receptor (SITR) (**CHAPTER 7**). Carp SITR is a type I protein with two extracellular Ig domains in a unique organisation of a N-proximal V/C2 (or I-) type and a C-proximal V-type Ig domain, devoid of a transmembrane domain or any intracytoplasmic signalling motif. Carp SITR is abundantly expressed in macrophages and is secreted upon stimulation with the protozoan parasite *T. borreli*. Overexpression of carp SITR in mouse macrophages resulted in activation of tyrosine phosphorylation-dependent intracellular signaling cascades and increased nitric oxide levels, suggesting a role for the SITR molecule in NO production. Knock-down of SITR protein expression in carp macrophages provided evidence for the involvement of carp SITR in *T. borreli*-induced NO production.

In **CHAPTER 8**, we discuss the importance of using highly purified ligand preparations when studying activation of receptors such as TLR2. Furthermore, we discuss the apparent suppression of p35 (IL-12) gene expression induced by protozoan infections and discuss the ability of protozoa to modulate signalling pathways and consequently inhibit IL-12 production. We put forward the hypothesis that fish TLR21, next to TLR9, could have a role as DNA receptor and discuss the functional consequences of an expanded SITR repertoire in teleost fish. Finally, we discuss our findings on innate immune receptors in the context of immune responses of carp to protozoan blood parasites.

Aanpassings- en ontduikings strategieën hebben het mogelijk gemaakt voor parasieten in de Orde Kinetoplastida aanwezig te blijven in bijna alle soorten vertebraten. Infecties met deze protozoë parasieten zijn niet alleen onder warmbloedigen wijdverbreid, maar ook bij koudbloedige vertebraten. In **HOOFDSTUK 1** introduceren we de hypothese van dit proefschrift dat macrofagen van karper (*Cyprinus carpio* L.) patroonherkenningsre septoren (PRR) hebben die essentieel zijn voor het herkennen van de protozoë parasieten *Trypanoplasma borreli* of *Trypanosoma carassii* and belangrijk zijn voor de ontwikkeling van een aangeboren immuunrespons.

Als een pathogeen de fysieke barrières eenmaal doorbroken heeft, kan het herkend worden door PRR op cellen van het aangeboren immuunsysteem en een hele serie van immuunreacties activeren die gericht zijn op het verwijderen van het pathogeen. Daarom spelen PRR een belangrijke rol in het detecteren van moleculaire structuren die uniek zijn voor pathogenen (dit wordt signaal 0 genoemd). Onder andere macrofagen kunnen antigenen afkomstig van pathogenen presenteren aan cellen van het adaptieve immuunsysteem (signaal 1) en cytokinen uitscheiden die het adaptieve immuunsysteem modelleren (signaal 2), wat leidt tot verwijdering van het pathogeen. Deze signalen zijn beide nodig om specifieke B en T lymfocyten te activeren. Adjuvanten worden ingedeeld in "signaal 1 facilitatoren" en "signaal 2 facilitatoren" (**HOOFDSTUK 2**) en kunnen door deze werkingen leiden tot een efficiënter design van vaccins, ook voor gebruik in aquacultuur.

Om de functionele gevolgen van de betrokkenheid van aangeboren immuunreceptoren te bestuderen, hebben we eerst een in vitro kweeksysteem ontwikkeld om relatief zuivere culturen van macrofagen te verkrijgen, afkomstig van de kopnier van karpers. Deze macrofagen, die het best vergeleken kunnen worden met macrofagen afkomstig van het beenmerg, behouden de mogelijkheid tot fagocytose en productie van radicalen (**HOOFDSTUK 3**), en zijn belangrijk voor onze verdere studies over de herkenning van PRR van protozoë parasieten. Tussen de verschillende klassen van PRR die in staat zijn een grote range van pathogeen geassocieerde moleculaire patronen (PAMP) te herkennen, is zonder twijfel de familie van de Toll-like receptoren (TLR) een heel belangrijke klasse van PRR.

Als eerste hebben we de rol van de karper TLR2-receptor in de herkenning van prototypische liganden in detail onderzocht. Transfectie van humane HEL 293 cellen met TLR2, bevestigde dat karper TLR2 kan binden aan LTA en PGN van *Staphylococcus aureus* en de synthethic triacylated lipopeptide Pam_3CSK_4, maar niet aan diacylated lipopeptide MALP-2 (**HOOFDSTUK 4**). Het gebruik van dezelfde liganden op karper macrofagen die TLR2 overproduceren, laat zien dat karper macrofagen hoge concentraties nodig hebben van LTA en PGN, minder sterk reageren (Pam_3CSK_4) of helemaal niet reageren (MALP-2) en dus een verschil laten zien in de immuunreactie van het TLR bevattende receptorcomplex tussen zoogdieren en karpers.

De levende protozoë parasieten *T. borreli* of *T. carassii* en GPI-ankers afkomstig van beide protozoë parasieten zijn bestudeerd als TLR2- ligand. Gedurende een immuunreactie, kan de balans tussen interleukine (IL)-12 (p35, p40) and IL-23 (p19, p40) afhangen van het

type PAMP dat herkend wordt door TLR2. Transfectie studies van HEK 293 and karper macrofagen hebben aangetoond dat PAMP afkomstig van *T. carassii*, antagonsiten zijn van karper TLR2 en p19- en p40c- genexpressie verhogen (**hoofstuk 5**). Gedurende met name *T. carassii* infecties hebben we een neiging waargenomen om p19- en p40c-genexpressie te induceren, wat de formatie van IL-23 suggereert. IL-17A/F2 genexpressie werd alleen bij *T. carassii* infecties waargenomen. We konden een duidelijke toename waarnemen van neutrofiele granulocyten uit de milt gedurdende *T. carassii* infectie, tesamen met een toename in genexpressie van metalloproteinase-9 en chemokines, hetgeen bewijs levert voor een Th17-achtige immuunrespons in reactie op infectie met *T. carassii*.

TLR9 is een belangrijke PRR die onder andere DNA-houdende ongemetyleerde CpG motieven detecteren die gevonden worden in bacterieel en protozoën (trypanosome) DNA. Het is aangetoond dat karper TLR9 een TLR7 signatuur heeft met 26 leucine-rijke herhalingen (LRR) en een uitgebreide flexibele lus in LRR15 (**Hoofdstuk 6**). Wanneer karper TLR9 getransfecteerd is in humane HEK 293 cellen, herkende het *E. coli* DNA, maar geen protozoe *T. borelli* DNA of enig andere van de geteste CpG motieven. Inhibitie van protease activiteit in karper macrofagen, verlaagde cytokine genexpressie na stimulatie met *E. coli* DNA. Dit suggereert de aanwezigheid van protease-afhankelijke TLR9 activatie routes voor karper TLR9.

Onderzoek van een cDNA repertoire, van karper macrofagen verrijkt met opgereguleerde genen in reactie op *T. borreli*, identificeerde een nieuwe immunoglobuline-achtige receptor in oplossing die we Soluble (oplosbaar) Immune-Type Receptor (SITR) genoemd hebben (**hoofdstuk 7**). Karper-SITR is een type I-eiwit met twee extracellulaire Ig- domeinen in een unieke organisatie van een N-eind V/C2 (or I-) type en aan C-eind V-type Ig- domein, zonder transmembraan domein of een intracytoplasmatisch signaal motief. Karper- SITR wordt overvloedig geproduceerd in macrofagen en is gesecreteerd na stimulatie met de protozoë parasiet *T. borreli*. Overproductie van karper-SITR in muis macrofagen resulteerde in activatie van tyrosine fosforylatie-afhankelijk intracellulaire signaal cascades and verhoogde nitriet oxide levels, wat suggereert dat het SITR molecuul een rol heeft in NO productie. Knock-down van de SITR-eiwitexpressie in karper macrofagen leverde bewijs voor de betrokkenheid van karper-SITR in NO productie geïnduceerd door *T. borreli*.

In **hoofdstuk 8** bespreken we het belang van het gebruik van ultra zuivere ligand preparaten wanneer activatie van receptors zoals TLR2 bestudeerd worden. Verder bespreken we de waarschijnlijke onderdrukking van p35 (IL-12) gen expressie geïnduceerd door protozoë infecties and bespreken de capaciteit van protozoën om signaal routes te moduleren en als gevolg IL-12 productie te kunnen remmen. We formuleren de hypothese dat in vissen TLR21, naast TLR9, een rol kan spelen als DNA-receptor en bespreken de functionele gevolgen van een uitgebreid SITR-repertoire in beenvissen. Tot slot bespreken we onze resultaten over aangeboren immuunreceptoren in de context van immuun responsen van karper tegen protozoë bloed parasieten.

Devido às adaptações e estratégias de evasão, os parasitas pertencentes à Ordem Kinetoplastida são capazes de persistir na maioria dos vertebrados, quer endotérmicos quer ectotérmicos. No **Capítulo 1** introduzimos a hipótese da presente tese em que macrófagos da carpa (*Cyprinus carpio* L.) possuem receptores (PRR) essenciais para a detecção dos protozoários *Trypanoplasma borreli* e *Trypanosoma carassii*, sendo estes receptores fundamentais para a ocorrência de respostas de immunidade inata.

Quando um patógeno ultrapassa as barreiras físicas, pode ser reconhecido por PRR presentes nas células do sistema imune inato, iniciando uma série de respostas imunes com objectivo na remoção desse patógeno. Os PRRs têm assim um papel importante na detecção de estruturas moleculares únicas dos patógenos (fornecendo o "sinal 0"). Os macrófagos, entre outros, apresentam os antigénios derivados de patógenos a células do sistema imune adaptativo ("sinal 1"), e secretam citoquinas que influenciam o sistema imune adaptativo ("sinal 2"), culminando na remoção do patógeno. Os dois últimos sinais são ambos importantes para a activação de linfócitos B e T. Adjuvantes podem ser categorizados como "mediadores de sinal 1" e/ou " mediadores de sinal 2" (**Capítulo 2**), levando a uma maior eficiência na contrucção de vacinas, também na práctica de aquacultura.

Para estudar as consequências funcionais da activação de receptores desenvolvemos um sistema in vitro para obter culturas relativamente puras de macrófagos derivadas do pronéforo da carpa. Estes macrófagos, comparavéis aos macrófagos derivados da medúla óssea, retêm a capacidade de fagocitar e produzir radicais (**Capítulo 3**), e foram preponderantes no estudo de PRRs responsáveis pela detecção de protozoários. Dentro das distintas classes de PRRs capazes de reconhecer os differents PAMPs, a família de Toll-like receptors (TLR) é, sem dúvida, uma importante classe de PRRs.

Investigámos, em detalhe, o papel do TLR2 da carpa no reconhecimento de ligandos prototípicos. A transfecçaõ de células de HEK293 com TLR2 corroborou a capacidade do TLR2 da carpa de se ligar ao LTA e PGN de *Staphylococcus aureus* e ao lipopéptido triacetilado sintético Pam_3CSK_4, mas não ao lipopéptido diacetilado MALP-2 (**Capítulo 4**). O uso dos mesmos ligandos em macrófagos da carpa que sobreexpressam TLR2 indicaram que macrófagos da carpa necessitam de concentrações altas de LTA e PGN, têm uma resposta menos forte ou não reagem (MALP-2), evidenciando as diferenças da resposta imune envolvendo o complexo TLR2 entre mamíferos e carpa.

Os protozoários *T. borreli* ou *T. carassii* e âncoras de GPI derivados de ambos os protozoários foram examinados como ligandos de TLR2. Durante uma resposta imune, o balanço entre interleuquina 12 (IL)-12 (p35, p40) e IL-23 (p19, p40) pode depender da natureza do PAMP reconhecido pelo TLR2. Estudos de transfecção com HEK 293 e macrófagos da carpa demonstraram que PAMPs derivados de *T. carassii* são agonistas do TLR2 da carpa, promovendo a expressão génica de p19 e p40c (**Capítulo 5**). Durante infecções com *T. carassii* em particular, observámos a tendência em induzir a expressão génica de p19 e p40c, sugerindo a formação de IL-23. Expressão génica de IL-17A/F2 foi observada somente durante infecções com *T. carassii*. Detectámos um incremento no número de neutrófilos do baço em conjunto com um aumento da expressão génica da

metaloproteinase-9 e quimoquinas, demonstrando a ocorrência de uma resposta imune tipo Th17 em carpa em resposta a infecções com *T. carassii*.

TLR9 é um importante PRR que detecta, entre outros, motivos de CpG DNA desmetilados presentes no DNA bacteriano e de protozoários (tripanossoma). TLR9 da carpa pertence à família TLR7 com 26 LRR e tem um loop flexível na LRR15 (**Capítulo 6**). TLR9 da carpa, quando transfectado em células humanas HEK 293, detectou DNA de *E. coli*, mas não DNA de *T. borreli* ou quaisquer motivos de CpGs. A inibição da actividade de proteases em macrófagos de carpa diminuiu a expressão génica de citoquinas após estimulação com DNA de *E. coli,* sugerindo que a activação de TLR9 da carpa é dependente de proteases.

A investigação de um repertório de cDNA de macrófagos de carpa enriquecidos com genes *up-regulated* em resposta a *T. borreli*, perimitiu a identificação de um novo receptor solúvel denominado SITR (**Capítulo 7**). O SITR da carpa é uma proteína tipo I com dois domínios Ig com uma organização única com um N-proximal do tipo V/C2 (ou I-) e um C-proximal com domínio Ig tipo V, Este receptor é desprovido de domínio transmembranar e de um motivo intracitoplasmático de sinalização. O SITR de carpa é abundamentemente expressado em macrófagos e é secretado após estimulação com o parasita protozoário *T. borreli*. Sobreexpressão do SITR da carpa em macrófagos de ratinho resultou na activação de cascatas de sinalização intracelulares dependentes da fosforilização de tirosina e do aumento dos níveis de óxido nítrico (NO), sugerindo um papel da molécula de SITR na produção de NO. *Knock-down* da expressão da protéina de SITR em macrófagos de carpa evidenciaram o envolvimento do SITR de carpa na produção de NO induzida por *T. borreli*.

No **Capítulo 8** discutimos a importância do uso de preparações de ligando extremamente puras aquando do estudo de activação de receptores como o TLR2. Analisamos a aparente supressão da expressão génica de p35 (IL-12) induzida durante infecções com protozoários e discutimos a capacidade de protozoários em modular vias de sinalização e consequentemente inibir a produção de IL-12. Propomos ainda a hipótese em que o TLR21 de peixe, juntamente com TLR9, poderá ter um papel como receptor de DNA e discutimos as consequências funcionais de um reportório mais extensivo em teleóstos. Finalmente, avaliamos os nossos receptores no contexto de respostas imunes de carpa para parasitas protozoários.

" Thousands of candles can be lighted from a single candle, and the life of the candle will not be shortened. Happiness never decreases by being shared."

Buddha

Acknowledgements

This "PhD journey" was an incredible personal challenge that only became possible and more pleasant because of the help, care and contributions of many people.

Geert, you were my first supervisor in science. We have been working together for almost six and half years, first as a student, then as researcher and finally as a PhD student. During all these years, I learned a lot and I certainly developed a passion for the "fish world". Thank you for all the support in carrying out new ideas, for all the brainstorming discussions and for all the opportunities you created for me. One thing became clear to me, if you want, you can make dreams come true.

Huub, you have endless energy and your enthousiasm for science is very contagious. I'm very thankful for all your encouragement and votes of confidence. Our lively discussions and your critical comments just make science even more fun. We share the passion to work in the lab "where things happen" so I'm hoping to find you also there more often. We also encountered ourselves in challenging situations and I appreciate your creative solutions. Above all, I'm very happy to have decided in 2004 to come to CBI and even happier that I can stay longer.

My dearest Anja and Nico, you are a lovely duo and I have the feeling I was adopted by you two! You are genuine, honest and you have huge hearts. Nico, your prompt help and your endless solutions made my life many times easier. Anja my paranymph, I'm very thankful for all the times we spent in the "dark", your sence for perfection and for all the beautiful pictures you took. Above all I always enjoyed the times we spent all together, because you are both great fun and I'm very grateful that your doors were always open. Magda, my Magdalena, your were my roommate for two years, and how lovely was that! Thank you for our daily discussions, the support and cooperation in the lab and the refreshing advices. You have been always a great source of inspiration to me in the lab and outside. I will also never forget my "Eastern" European visit. I loved your country and your food and I will definetely visit you again. I feel very proud to have had you as a roommate, friend and in "the big day" as a paranymph.

Tosquita, my forever roommate. I feel we "grew" together in science, you with your big and milky animals and me with my small and swimming ones. We shared so much and i want to keep doing. You taught me how important it is to be assertive and fight for what you deserve, however, I still think I need some more lessons. Many times, your warm words and your caring smile made the difference. Thank you for all, my friend. Nathalie, our newest Zoolander, you always took so good care of me! I really enjoyed our discussions about lab and experiments, I definetely think we should keep having those. I thank you for being such a good listener, for always "pumping" me with energy and confidence and for being such a lovely friend. I look forward for our future times together.

Trudi, your interest in new ideas and your endless patience for solving problems were crucial for me to arrive at this stage. I deeply appreciate all your help. Marleen, you have been spoiling me so much all these years. I thank you for all the effort, help and compromises you did. Some experiments I could have not done without you and these were just much more fun with you. Adrie "my collegatje", Beja, Maria de B. I missed you all a lot! But the memories, those are great. Hilda, my dear neighbour, you always knew when to cheer me up. Virgil, I enjoyed a lot writing the book chapter with you and all the joined discussions. Thank you for all your suggestions, I learned a lot from those too.

A great word of appreciation for all the tips, discussions and advices to all the staff members, colleagues and fellow-students at CBI and EZO. I would like to thank, in particular, Anders, Edwin, Ellen, Henk, Inge, Jan, Joop, Jos, Karen, Lidy, Lieke, Maria, Mark, Prescilla, Ruth and Yvonne. Your experience and knowledge were many times valuable tools to successfully reach my goals. A special thanks to Maaike, you helped me in making "my first steps" in the lab. I feel we share our love for "The cells" and for the sometimes forgotten *T. carassii*. I learned a lot from the time we worked together.

Acknowledgements

A BIG thanks to all my students, Jochem, Bas, Natalia, Lisa and Laura. To be a supervisor of MSc students was also a challenge for me but I feel I learned from each of you and I totally loved it. I am very grateful for your great help and dedication.

Maria José, you worked so much! We had a great chemistry in the lab and many times I had the feeling we worked together as one. Some of my chapters were only possible because you were always there to help me. Obrigada. Mas acima de tudo, fostes sempre uma excelente amiga. Agradeço-te por muitas vezes teres sido um ouvinte e por todas as palavras de motivação. As nossas experiências culinárias foram sempre um sucesso e as nossas festas inesquecíveis. No futuro, temos que repetir tudo isto e muito mais.

Danilo, Fabrizio, Gerco, Indra, João, Makoto, Marcel, Marian, Santiago and Silvia, I had the opportunity to share great and unforgettable times with each of you at CBI and outside. You were incredible friends throughout this journey! A very "big" hug from this friend of yours.

I would like to express my great gratitude and appreciation to all the members of the Laboratory of Cellular and Molecular Immunolgy in Brussels. Your hospitality was great. I would like to thank Geert and Reza, in particular, for all the supervison and great support. I feel my "SITR" was born in Belgium so i hope we can keep our cooperation in the future.

I also had the opportunity to visit the Scottish Fish Immunology Research Centre in Aberdeen. A very BIG thanks to all the members and specially to Charo and Patricia for spoiling me with great food and for the great hospitality. Steve, I learned a lot with you in a week. Thank you so much for all your patience, time and great advices. I hope you have more of those because I intend to keep collaborating with you hereafter.

All this adventure would not have ever been possible if I would not have had the best H87 corridor-mates ever. Andreas, Anne-Douwe, Arthur, Chris, Harm, Jan-Wouter Jens, Klaas, Kostas, Susanne and Wim, you raised me, fed me, put me to sleep and, above all, were many times my family. When I think back at our adventures I cannot avoid getting a great smile on my face. You too embraced my dream like it was yours and always understood when I needed a hug or a push. A big "boks"to my dear Amar, Belg, Menno and Uju. You have always been great to me.

Ma e Pa, eu sei que foi muitas vezes difícil lidar com a minha ausência e entender este meu sonho. Eu agradeço todos os sacrifícios, compreensão e mimo com esta filhota. O vosso incondicional apoio e entusiasmo tornaram este meu sonho uma realidade. Obrigada por tudo isto e muito mais. Lipe, eu sei que posso e poderei sempre contar contigo. Nunca poderia ter desejado um melhor mano. Quero também enviar muitos beijinhos às minhas queridas Família Sampaio e Família Ribeiro. Um beijo especial aos meus queridos Padrinhos e Tio Jorge por todo o apoio e carinho.

Lieve Familie Touw en Familie van der Veen, ook jullie hebben mij geadopteerd. Ik ben heel erg dankbaar voor jullie steun en toeverlaat. Gonny en Jacques, jullie stonden altijd voor mij klaar met een grote lach, gezelligheid en heerlijke maaltijden. In moeilijke tijden hebben jullie altijd een oplossing voor mij gevonden. Wij hebben zo veel goed herinneringen en ervaringen gedeeld. Ik ben er zeker van dat ik de best schoonouders heb.

Wouter, my corridor-mate, my friend, my man. We both know how this journey was very intensive. Arriving home and finding myself in the comfort of your embrace was always encouraging for the days to follow. I am extremely grateful for all the constant compromises, the endless understanding and the never-ending love you gave to me. You are special, you have the talent to make others feel special. You "rock my world".

Carla

"And more, much more than this, I did it my way."

Frank Sinatra

Personalia

CURRICULUM VITAE

Carla Mónica Sampaio Ribeiro was born in Oporto on 12th December 1982. In 2000, after graduating at the Scientific Gymnasium "Ciências Naturais" in Vila Nova de Gaia, she started her study in Biochemistry at the Science Faculty, Oporto University. In 2004, during the last six months of her study she was an "Erasmus exchange student" at the Cell Biology and & Immunology Group at Wageningen University. She performed her thesis entitled: "Macrophage polarization during parasite infections" under supervision of Dr. Geert Wiegertjes and Prof. Huub Savelkoul. From November 2004 till August 2005 she was enrolled in an European, Marie Curie Research Training Network entitled "Integrated approach to the innate immune response to parasites in fish (PARITY) at the Cell Biology and & Immunology Group. She was awarded a PhD scholarship by the Portuguese foundation "Fundação para a Ciência e Tecnologia". In January 2006, she started as PhD student at the Cell Biology and & Immunology Group under supervision of Dr. Geert Wiegertjes and Prof. Dr. Ir. Huub Savelkoul. Currently, she has a temporary position at the Cell Biology and & Immunology Group combining education and research work.

List of publications

Published papers:

Ribeiro, C.M.S.; Pontes, M. J. S. L.; Bird, S.; Chadzinska, M.; Scheer, M.; Verburg-van Kemenade, B. M. L.; Savelkoul, H. F. J.; Wiegertjes, G. F. (2010) Trypanosomiasis-induced Th17-like immune response in carp. *PloS One, In press*

Ribeiro, C.M.S.; Hermsen, T.; Taverne-Thiele, A.J.; Savelkoul, H.F.J.; Wiegertjes, G.F. (2010) Evolution of recognition of ligands from Gram-positive bacteria: similarities and differences in the TLR2-mediated response between mammalian vertebrates and teleost fish. *Journal of Immunology* 184- p. 2355-2368

Ribeiro, C.M.S. and Schijns, V.E.J.C. (2010) Immunology of Vaccine Adjuvants. *Vaccine Adjuvants,* Methods in Molecular Biology, Gary Davies (Editor), Humana Press Inc.

Ruszczyk, A.; Forlenza, M.; Joerink, M.; **Ribeiro, C.M.S**.; Jurecka, P.M.; Wiegertjes, G.F. (2008)*Trypanoplasma borreli* cysteine proteinase activities support a conservation of function with respect to digestion of host proteins in common carp. *Developmental and Comparative Immunology* 32 (11). - p. 1348 - 1361.

Joerink, M.; Forlenza, M.; **Ribeiro, C.M.S.**; Vries, B.J. de; Savelkoul, H.F.J.; Wiegertjes, G.F. (2006) Differential macrophage polarisation during parasitic infections in common carp (*Cyprinus carpio* L.). *Fish and Shellfish Immunology* 21 (5). - p. 561 - 571.

Joerink, M.; **Ribeiro, C.M.S.**; Stet, R.J.M.; Hermsen, G.J.; Savelkoul, H.F.J.; Wiegertjes, G.F. (2006) Head kidney-derived macrophages of common carp (*Cyprinus carpio* L.) show plasticity and functional polarization upon differential stimulation. *Journal of Immunology* 177 - p. 61 - 69.

Awarded conference publications:

Ribeiro, C.M.S.; Savelkoul, H.F.J.; Wiegertjes, G.F. (2009) *Immune responses of carp against parasites.* In: Wageningen Institute of Animal Sciences 2009, Wageningen, The Netherlands, 12th March, 2009 - *Best oral presentation*

Ribeiro, C.M.S.; Savelkoul, H.F.J.; Wiegertjes, G.F. (2008) *An investigation into parasite PAMPs activating carp macrophages via TLR2-ROS signaling*. In: Book of Abstracts 13th Congress of Polish Society of Experimental and Clinical Immunology - Central European Journal of Immunology 33 - p. 169, 14-17 May, 2008 *Best oral presentation and Young & Promising Scientist award*

TRAINING AND SUPERVISION PLAN

THE BASIC PACKAGE

WIAS Introduction Course, Wageningen, The Netherlands	2006
WIAS Course Broaden your Horizon Course, Wageningen, The Netherlands	2007
Subtotal Basic Package	**3 credits***

SCIENTIFIC EXPOSURE

International conferences

ISDCI[1], Charleston, USA	2006
NOFFI[2], Stirling, Scotland, UK	2007
Polish Society of Experimental and Clinical Immunology, Krakow, Poland	2008
ISDCI[1], Prague, Czech Republic	2009

Seminars and Workshops

WIAS seminar Macrophage polarization, Wageningen, The Netherlands	2006
WIAS seminar Immune responses to viruses, Wageningen, The Netherlands	2007
WIAS seminar Corticosteroid receptors in common carp, Wageningen, The Netherlands	2008
WIAS seminar Multidisciplinary study of allergy, Wageningen, The Netherlands	2008
WIAS seminar Fishing for Immunity, Wageningen, The Netherlands	2008
WIAS seminar Of fish and men: curiosities of the immune system, Wageningen, The Netherlands	2009
NCLMS[3] Symposium New Frontiers in Pattern Recognition Receptors, Nijmegen, The Netherlands	2009
WIAS Science Day	2006-10

Presentations

Polarization of carp macrophages: differential receptor expression (Oral) Charleston, ISDCI[1]	2006
A search for PAMPs stimulating carp macrophages (Poster) Stirling, NOFFI[2]	2007
An investigation into parasite PAMPs activating carp macrophages via TLR2-ROS signaling (Oral) Krakow, Polish Soc. of Exp. and Clinical Immunology	2008
TLR2 as pattern recognition molecules of parasites in carp (Oral) ISDCI, Prague, ISDCI[1]	2009
Immune response of carp against parasites (Oral) Wageningen, WIAS Science Day	2009
Subtotal Scientific Exposure	**14 credits***

In-Depth Studies

Disciplinary and interdisciplinary courses

NVVI Course Lunteren, The Netherlands	2006
Fish Vaccination Workshop, Wageningen, The Netherlands	2006
Post Graduate Course Immunology, Utrecht, The Netherlands	2007
WIAS Course ELISA: basic understanding and trouble shooting, Wageningen, The Netherlands	2007
Fish immunology Workshop, Wageningen, The Netherlands	2008

Advanced statistics courses

WIAS Course Design of Animal Experiments, Wageningen, The Netherlands	2007
Subtotal In-Depth Studies	**6.4 credits***

Statutory Course

Laboratory Animal Sciences, Utrecht, The Netherlands		2007
	Subtotal Statutory Course	**3 credits***

Professional Skills Support Courses

WIAS Course Career Coaching, Wageningen, The Netherlands		2007
Project & Time Management, Wageningen, The Netherlands		2007
Techniques for Scientific Writing and Presenting a Scientific Paper, Wageningen, The Netherlands		2008
NWO[4] Talent Day, Utrecht, The Netherlands		2009
	Subtotal Professional Skills Support Courses	**3 credits***

Research Skills Training (External training periods)

Performing Substractive Hibridization at Dr. Geert Raes Lab, Vrije Universiteit Brussel, Brussels, Belgium		2006
Performing Bioinformatics at Dr. Steve Bird Lab, University of Aberdeen, Aberdeen, Scotland, UK		2009
	Subtotal Research Skills Training	**2.5 credits***

Didactic Skills Training

Supervising practicals and excursions

Practicals Fish Immunology Workshops	2006-07
Practicals Comparative Immunology	2007
Practicals Human & Veterinary Immunology	2008-09

Supervising theses

1 minor MSc thesis		2007
4 major MSc theses		2007-09
	Subtotal Didactic Skills Training	**17.5 credits***

Total number of credit points	**49.4 credits***

Herewith the WIAS Graduate School declares that the PhD candidate has complied with the educational requirements set by the educational Committee of WIAS.

* According to the ECTS (European Credit Transfer and Accumulation System), one credit point represents a normative study load of 28 hours

[1] International Society for Developmental & Comparative Immunology

[2] Nordic Society for Fish Immunology,

[3] Nijmegen Centre for Molecular Life Sciences

[4] Nederlandse Organisatie voor Wetenschappelijk Onderzoek

The research described in this thesis was financially supported by the portuguese foundation Fundação para a Ciência e Tecnologia under Grant agreement number SFRH/21522/2005

Cover design: Carla M. S. Ribeiro and Anja J. Taverne-Thiele
Layout: Carla M. S. Ribeiro

The thesis was printed by Wöhrmann Print Service.

Financial support from Wageningen University, Fundação para a Ciência e Tecnologia and Crossbeta Biosciences B.V. for printing this thesis is gratefully acknowledged.